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(54) **NEW NUCLEIC ACID BASE PAIRS**

NEUE NUKLEINSÄURE-BASISPAARE

PAIRES DE BASES D'ACIDES NUCLÉIQUES

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Description**Background and Summary of the Invention**

[0001] This invention relates to non-natural bases and base pairs. The bases are an expansion of a DNA based encoding system. For example, the system is based on Watson-Crick type Acceptor:Donor hydrogen bond base pairing patterns.

[0002] Mutually complementary pairs of oligomers, such as DNA and PNA, are used in countless applications as molecular labels/recognizers and probes (see e.g. WO9220703 or WO03080857). The diversity of the 4 naturally occurring nucleobases, A, C, G, and T allows a multitude of diverse labels to be constructed (a 10' mer comes in $4^{10} = 1$ million versions). Furthermore, the labels are recognized in a modular fashion by Watson-Crick base pairing, where A:::T, G:::C and vice versa, i.e., from the label follows the complementary strand.

[0003] The use of DNA and PNA, however, is limited by some inherent drawbacks. For example, DNA, while having several desirable properties such as easy enzymatic or chemical synthesis, is poly anionic with each base contributing with a negatively charged phosphate moiety. Thus, while DNA will hybridize to its complement with high affinity and specificity, it will also bind unspecifically to any poly cationic species such as many proteins, metal ions and surfaces.

[0004] Since PNA is a non charged polypeptide oligomer, it does not exhibit such unspecific affinity for poly cations or poly anions. However, the lack of charge gives generally lower solubility and increases inter and intra-molecular interactions and tends to make PNA highly self-aggregating.

[0005] Also limiting the use of a base pairing oligomer is its self-structure. DNA strands of even moderate length will form intramolecular hairpins and other complex structures that basically hide the bases from recognition by the complementary oligomer. This is especially pronounced for PNAs where charge-charge repulsions do not tend to straighten the backbone. The self structure may significantly slow down the rate of and can even ultimately prevent the desired hybridization between complementary strands.

[0006] The problem may be further augmented when for amplification purposes more than one label-PNA/DNA is attached to the same probe. Intermolecular interactions may then contribute to aggregation and formation of undesirable complexes that are not readily available for recognition by the complementary strand.

[0007] Additionally, in most samples, more than one potential target will be present. For example, in almost any bioassay, there will be RNA and/or DNA present. Thus, the probes may interact specifically yet undesirably with RNA and DNA present in the sample producing false results. Even PNA, which by merit of its higher self-affinity, than DNA/RNA might specifically recognize complementary PNA even in the presence of competing DNA/RNA, is limited by a very slight difference in discrimination.

[0008] The inventor has found that by increasing the number of bases, complementary labels with varying mutual affinity can be obtained. The affinity may also be modulated by the use of strong 3 hydrogen bond G:::C-type base pairs over weaker 2 hydrogen bond A::T-type base pairs. This is in sharp contrast to molecular recognition via proteins where there are no similar simple rules governing complex interactions. It has also been discovered that increasing the number of bases may overcome at least one of the drawbacks outlined above.

[0009] Compositions are disclosed that comprise more than one non-natural base. Bases used herein include the natural bases, A, G, T, C, and U, and the purine-like and pyrimidine-like molecules disclosed herein and any other non-natural base that may interact with another base via a Watson Crick-type, wobble, and/or Hoogsteen-type hydrogen bonding geometry. The bases may be used in molecular entities, such as PNA oligomers where the bases are attached to a backbone.

[0010] The non-natural bases of the invention may be chosen from the purine-like molecules and the pyrimidine-like molecule disclosed herein. In one embodiment, a composition is disclosed comprising at least one molecular entity, which comprises a set of molecules, each comprising at least one non-natural base, such as, for example, at least one purine-like molecule and at least one pyrimidine-like molecule. The molecular entity, may, for example, be double stranded and the backbone of each strand may be the same or different.

[0011] The interaction between the at least one purine-like molecule and the at least one pyrimidine-like molecule may comprise two or three hydrogen bonds. For example, the interaction between the at least one purine-like molecule and the at least one pyrimidine-like molecule may comprise an interaction chosen from a Watson Crick-type wobble and a Hoogsteen-type hydrogen bonding geometry. Bases on an entity may also be chosen and/or paired with other bases where there are single interactions, no interactions or repulsions. This may enable one to fine tune the affinity of the bases for one another.

[0012] Compositions of the invention may be used in a method of labeling a biological substance comprising the step of binding at least one composition of the invention to the biological substance. Methods of making the compositions of the invention and methods of designing probes using the non-natural bases of the invention are also disclosed.

Brief Description of the Figures**[0013]**

Figure 1 (a-r): Examples of natural and non-natural bases and their names and symbols. Where:

R1 denotes the attachment point to the backbone.

R2 is, for example, substituents in the 8-position of purines: such as hydrogen, halogens, or other small substituents i.e., methyl, ethyl.

R3 is, for example, substituents on hydrogen bonding exocyclic amino groups on bases other than cytosine: such as hydrogen, methyl, ethyl, acetyl.

R4 is, for example, substituents that face a carbonyl in place of an aminogroup: such as hydrogen, fluorine and chlorine.

R5 is, for example, substituents in the 5-position of pyrimidines: for example, fluorofors, hydrogen, halogens, and substituted and unsubstituted groups of C1-C20. This position, for example, allows bulky substituents, if desired.

R6 is, for example, substituents on the hydrogen bonding exocyclic amino group of cytosine. This position also allows bulky substituents, for example, alkyl, acyl, and substituted and unsubstituted groups of C1-C20.

Figure 2 (a-q): Examples of base pairs of the invention. Where:

R1 denotes the attachment point to the backbone.

R2 is, for example, substituents in the 8-position of purines: such as hydrogen, halogens, or other small substituents i.e., methyl, ethyl.

R3 is, for example, substituents on hydrogen bonding exocyclic amino groups on bases other than cytosine: such as hydrogen, methyl, ethyl, acetyl.

R4 is, for example, substituents that face a carbonyl in place of an aminogroup: such as hydrogen, fluorine and chlorine.

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R6 is, for example, substituents on the hydrogen bonding exocyclic amino group of cytosine. This position also allows bulky substituents, for example, alkyl, acyl, and substituted and unsubstituted groups of C1-C20.

Figure 3: Examples of the pairing of base pairs of Figure 1 and 2 with the number of hydrogen bonds involved in the pairings.

Figure 4: An Example of 4 sets of probes designed using the base of the invention. Beginning with the set of probes made of 6'mers of G and Gs (G/Gs probes), a set of probes made of 6'mers of C and P may be designed such that each probe only recognizes one of the G/Gs probes. Another series of 6'mers of C and P may be designed such that some probes will recognize two of the three G/Gs probes. While a 6'mer of P will recognize all three G/Gs probes. Figure 5: Illustrates the difference in energy barrier to complex formation if the stable structures of the oligomers pre-complexation are aligned and/or by preparing oligomers that require little reorganization to reach the complexation transition state because of lack of a stable self structure (Figure 5).

Figure 6: Illustrates the interactions between each of the 18 bases shown in Figure 1: 3 refers to three hydrogen bonds being present between the bases; 2 refers to two hydrogen bonds being present between the bases; 1 is the presence of one hydrogen bond; and X is a repulsion or no H bonding between the pairs. There are 3 three bond base pairs, 12 two bond base pairs, and 2 single bond base pairs.

Figure 7: Illustrates further examples of bases and base-pairs, specifically, interactions between a series of base-

pairs involving Xanthine, 2-thio-xanthine, and 6-thio-xanthine. 4-aminopyrimidine-5-yl can pair with both Xanthine and 6-thio-xanthine, while 2-aminopyrimidine-5-yl can pair with both Xanthine and 2-thio-xanthine. 2,4-diaminopyrimidine-5-yl only pairs with xanthine.

Detailed Description of the Invention

Definitions

[0014] In the descriptions below the following definitions apply.

[0015] A linker includes any molecule that may help to join other atoms, molecules, or functional groups together through chemical bonds. A linker may include, for example, any molecule connecting any one of the bases of formula (1)-(16) to a backbone or any molecule that is serves to join another substance to any base or backbone.

[0016] A backbone may be any type of polymer including polyamide (e.g., peptide) backbones and sugar-phosphate backbones.

[0017] As used herein, the terms base and nucleobase refer to any purine-like or pyrimidine-like molecule that may be comprised in a nucleic acid or nucleic acid analog.

[0018] A non-natural base, as used herein, means any nucleobase other than: Adenine, A; Guanine, G; Uracil, U; Thymine, T; Cytosine, C. A non-natural base may also be referred to as a nucleic acid analog with sequences or oligimers, such as two or more of these bases referred to as nucleic acid analog segments.

[0019] A group that is optionally substituted may include a substitution with a group chosen from hydroxy, halogen, and -SH.

[0020] A molecular entity includes isolated base pair; oligomer entities ; polymer entities; double strand entities made of polymers, oligomers, or both; triple strand entities made of polymers, oligomers or both; quad strand entities made of polymers, oligomers or both; single or multi-strand entities made of polymer, oligomer, or both, interacting with isolated molecules; single or multi-strand entities made of polymer, oligomer, or both which includes with folds (such as loops, hairpins, and bulges); helical or helical-like entities and topoisomers thereof, coil or coil-like entities and topoisomers thereof, superhelical entities (such as toroidal helix, or interwound helix) and topoisomers thereof, nicked helix or nicked superhelix entities and topoisomers thereof, intercollated helix or superhelix entities and topoisomers thereof, right-handed helical entities (such as B-type helices or A-type helices), left-handed helical entities (such as Z form helices) and topoisomers thereof.

[0021] Base pairs, as used herein, includes any type of hydrogen bonding interaction between two bases and their adjacent backbone, including, but not limited to, geometry such as Watson-Crick, wobble, and Hoogsteen.

[0022] Double stranded encompasses a double strand of any type of polymeric backbone and encompasses at least PNA, RNA, and DNA polymer backbones, and includes, but is not limited to, any type of hydrogen-bonded base-pairing geometry such as Watson-Crick, wobble, and Hoogsteen.

[0023] Bind or hybridize, and like terms, when referring to natural or non-natural bases includes the formation of base pairs and/or double strands.

[0024] Set of, as used herein means at least two of an item.

[0025] A probe, as used herein, includes any molecular entity that is capable of recognizing a target substance present in a sample.

[0026] As used herein, all numbers are approximate, and may be varied to account for errors in measurement and rounding of significant digits.

Non-natural Bases

[0027] Figure 1 provides examples of non-natural bases. Figure 7 provides additional examples. The invention describes a general method or principle of generating a diversity of bases and base pairs. For example, the amino group/"h" and carbonyl/thiocarbonyl on either the purine or on the pyrimidine are used to pair the bases. The hydrogen bond in question is either facing the minor groove or with the hydrogen bond in question facing the major groove.

[0028] Similar to natural bases A, C, G, U, and T, some of non-natural bases disclosed herein are capable of forming stable three hydrogen bond base pairs between purines and pyrimidines, such as

Diaminopurine : Uracil; Guanine : Cytosine; isoGuanine : isoCytosine

[0029] Substituting one amino group with a Hydrogen or small Halogen (collectively "h") on one of the bases creates six types of bases :

2-amino-6-"h"-purines

6-amino-2-"h"-purines
 6-oxo-2-"h"-purines
 2-oxo-4-"h"-pyrimidines
 2-oxo-6-"h"-purines
 4-oxo-2-"h"-pyrimidines

These will form two hydrogen bond base pairs with non-thiolated and thiolated bases; respectively

2,4 dioxo and 4-oxo-2-thioxo pyrimidines
 2,4 dioxo and 2-oxo-4-thioxo pyrimidines
 4-amino-2-oxo and 4-amino-2-thioxo pyrimidines
 6-oxo-2-amino and 6-thioxo-2-amino purines
 2-amino-4-oxo and 2-amino-4-thioxo pyrimidines
 6-oxo-2-amino and 6-thioxo-2-amino purines

Whereas

4-oxo-2-thioxo pyrimidines will not pair with 2,6-diaminopurines or 2-amino-6-"h"-purines;
 2-oxo-4-thioxo pyrimidines will not pair with 2,6-diaminopurines or 6-amino-2-"h"-purines;
 4-amino-2-thioxo pyrimidines will not pair with 2-amino-6-thioxopurines or 2-"h"-6-thioxopurines;
 6-thioxo-2-amino purines will not pair with 4-amino-2-oxopyrimidines or 4-amino-2-thioxopyrimidines;
 2-amino-4-thioxo pyrimidines will not pair with 2-thioxo-6-aminopurines or 2-thioxo-6-"h"-purines; and
 6-thioxo-2-amino purines will not pair with 2-thioxo-6-aminopurines or 2-thioxo-6-"h"-purines.

[0030] In each of the above cases, two bases A and B have three mutual hydrogen bonds and form a very stable mutual pair. By substituting one of the hydrogen bonding amino groups on one of the bases, say A, with hydrogen or a small halogen, we get a new base C, and by substituting the facing carbonyl on B, we get a thiocarbonylated base D.

[0031] C will now pair with both B and D, forming two stable, two hydrogen bond pairs. A will however not pair with D (or other analogues of B with a thiocarbonyl facing an amino group in A). For example, 2-ThioUracil does not pair with Diaminopurine but pairs with Adenine, while Adenine pairs with both Uracil and 2-ThioUracil, i.e. these 4 bases follow the described pattern, A=Diaminopurine, B=Uracil, C=Adenine, D=2-ThioUracil.

[0032] These bases can be used to create molecular entities such as pairs of oligomers that pair well with each other and the number of hydrogen bonds or affinity of the base pairs can be used to fine tune affinity. In one embodiment, these entities result in reduced self structure and increased diversity with Uracils facing Diaminopurines or Adenines and Adenines further facing 2-Thiouracils, and 2-Oxo-pyrimidine pairs with both Guanine and ThioGuanine.

[0033] The bases of the invention can be defined as C, D, G, U from figure 1 and their derivatives, such as for example, one carbonyl-thiocarbonyl substitution (Cs, Gs, U2s, and U4s) or one deamination (A, isoA, I (inosine) and P (pyrimidinone)).

[0034] Also disclosed is an example of set of base pairing rules which one may use as a guide to design additional bases or molecular entities or probes that interact: 1) When part of complementary entities or oligomers, the base pairs obey Watson-Crick rules with hydrogen donors pairing with hydrogen acceptors, via hydrogen bonds, for example, pairing purine bases with pyrimidine bases. For example, C:G share three mutual hydrogen bonds and are thus very stable, while A:U share two mutual hydrogen bonds and contribute to complex stability.

2) Thiocarbonyls, such as on Cs, due to their increased size, repel bases with facing aminogroups, despite two other mutual hydrogen bonds (disallowing, for example, D:U2s and D:U4s) but may pair via two hydrogen bonds with bases that have a hydrogen facing the thiocarbonyls, allowing additional base pairs that also contribute to complex stability, such as Cs:I.

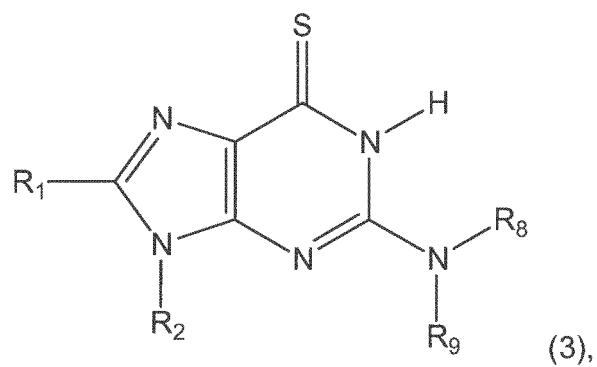
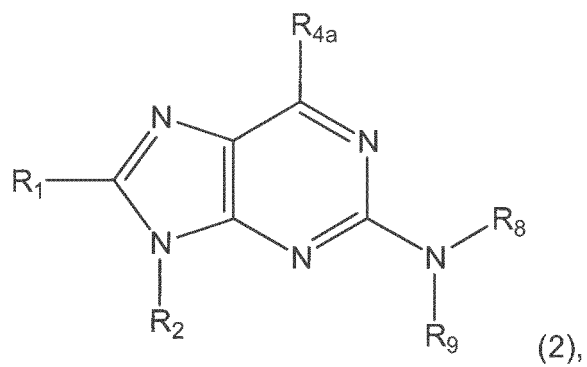
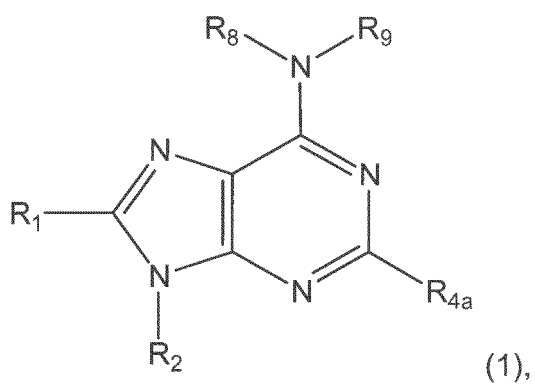
3) Some base pairs, such as I:P, only share a single central hydrogen bond, such as I:P, and is expected to be significantly weaker.

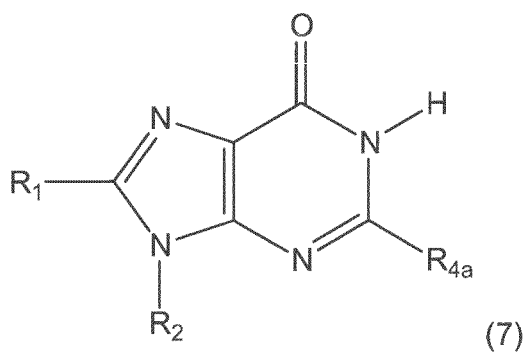
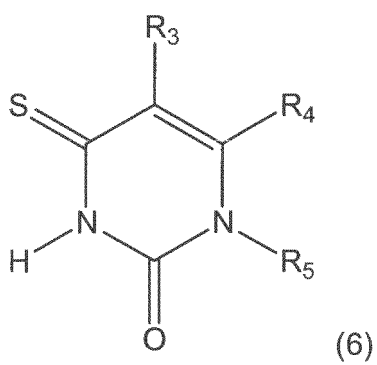
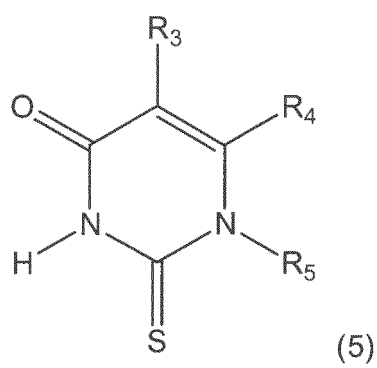
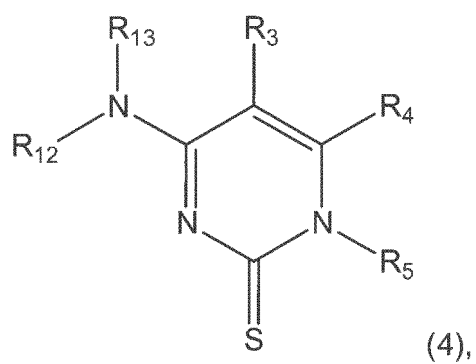
[0035] These rules provide an information encoding system that is expanded in comparison to use of natural base in that bases pair with either one (Cs, Gs, U2s, U4s, D), two (A, IsoA, C, G) or three other bases (I, P, U) with the same (A:U2s = A:U) or different affinity (D:U > A:U). Thus, for example, within the same reading frame the system allows multiple different PNAs to be recognized with the same affinity by one other PNA complementary to all of the PNAs, yet also allow each PNA to be recognized with unique specificity by other complementary, yet different PNAs.

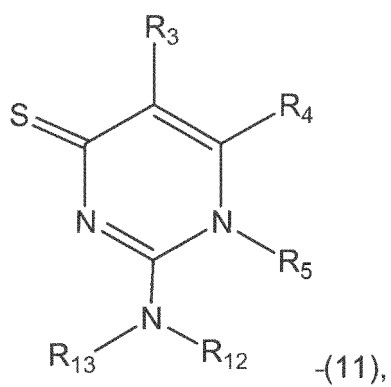
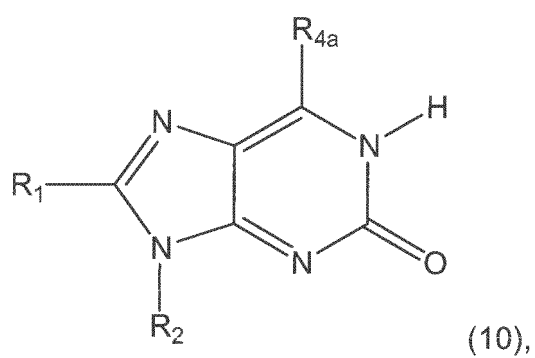
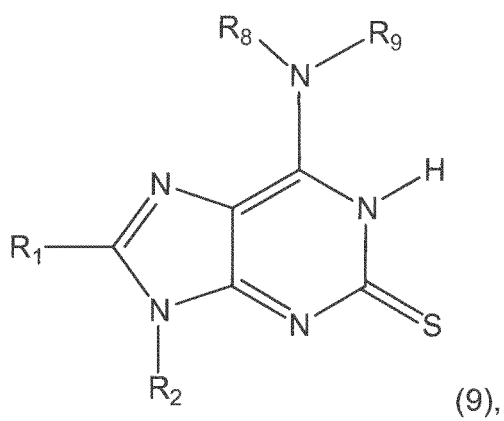
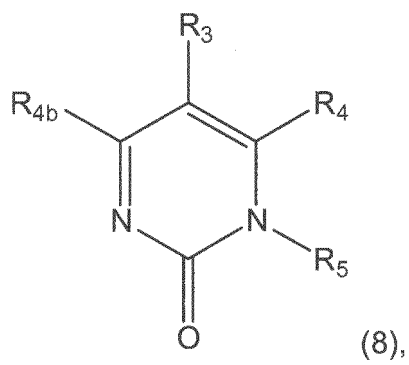
[0036] The non-natural bases of the invention can be made using synthesis techniques well known in the art. Examples are provided in the examples section below.

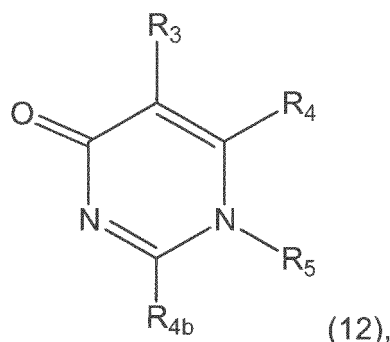
Molecular Entities Defined from a Single Base

[0037] Disclosed herein are compositions comprising molecular entities which are a pair of PNA oligomers, wherein each of the PNA oligomers comprises at least one molecule chosen from:



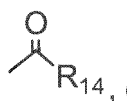






wherein

R_1 is chosen from hydrogen, halogen, optionally substituted (C₁-C₄)-alkyl, -NR₆R₇, optionally substituted (C₁-C₄)-alkoxy, optionally substituted (C₃-C₆)-cycloalkyl, linker, backbone, and linker bound to a backbone;
 R_2 is chosen from hydrogen, optionally substituted (C₁-C₄)-alkyl, -NR₆R₇, optionally substituted (C₁-C₄)-alkoxy, optionally substituted (C₃-C₆)-cycloalkyl, linker, backbone, and linker bound to backbone;
 R_3 is group chosen from hydrogen, halogen, optionally substituted (C₁-C₂₀)-alkyl, -NR₆R₇, optionally substituted (C₁-C₂₀)-alkoxy, optionally substituted (C₃-C₈)-cycloalkyl, linker, backbone, and linker bound to backbone;
 R_4 is chosen from hydrogen, halogen, optionally substituted (C₁-C₄)-alkyl, -NR₆R₇, optionally substituted (C₁-C₄)-alkoxy, optionally substituted (C₃-C₆)-cycloalkyl, linker, backbone, and linker bound to backbone;
 R_{4a} and R_{4b} can be identical or different, and are chosen from hydrogen, fluorine and chlorine.
 R_5 is chosen from hydrogen, optionally substituted (C₁-C₄)-alkyl, -NR₆R₇, optionally substituted (C₁-C₄)-alkoxy, optionally substituted (C₃-C₆)-cycloalkyl, linker, backbone, and linker bound to backbone;
 R_6 and R_7 can be identical or different, and are chosen from hydrogen and optionally substituted (C₁-C₂₀)-alkyl;
 R_8 , R_9 , R_{10} , R_{11} , R_{12} , and R_{13} can be identical or different, and are chosen from hydrogen, halogen,



optionally substituted (C₁-C₂₀)-alkoxy, and optionally substituted (C₃-C₈)-cycloalkyl, and optionally substituted (C₁-C₂₀)-alkyl; and
 R_{14} is chosen from hydrogen, optionally substituted (C₁-C₂₀)-alkyl, optionally substituted (C₁-C₂₀)-alkoxy, and optionally substituted (C₃-C₈)-cycloalkyl;
 R_{15} and R_{16} are chosen from oxygen and sulfur;
 R_{17} and R_{18} are chosen from hydrogen and -NH₂.

[0038] In some exemplary embodiments, the at least one molecular entity is not the isolated at least one molecule.

[0039] In some exemplary embodiments, R_1 is chosen from hydrogen, halogen, methyl and ethyl.

[0040] In some exemplary embodiments, R_2 is not a phosphate-ribonucleotide backbone, and is not a phosphate-deoxyribonucleotide.

[0041] In some exemplary embodiments, R_1 , R_3 , and R_4 are each is hydrogen.

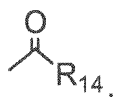
[0042] In some exemplary embodiments R_3 is chosen is chosen from optionally substituted (C₁-C₂₀)-alkyl, -NR₆R₇, optionally substituted (C₁-C₂₀)-alkoxy, optionally substituted (C₃-C₈)-cycloalkyl, and linker.

[0043] In some exemplary embodiments, R_2 and R_5 can be identical or different and are chosen from linker, backbone, and linker bound to backbone.

[0044] In some exemplary embodiments the backbone is chosen from a polyamide backbone and a sugar-phosphate backbone.

[0045] In some exemplary embodiments, R_1 , R_3 , and R_4 each is hydrogen; R_2 and R_5 can be identical or different radicals and are chosen from backbone and linker bound to backbone; and the backbone is chosen from a polyamide backbone and a sugar-phosphate backbone.

[0046] In some exemplary embodiments R_8 , R_9 , R_{10} , R_{11} , R_{12} , and R_{13} can be identical or different, and are chosen from hydrogen, halogen,

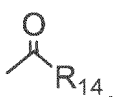


and optionally substituted (C₁-C₄)-alkyl.

[0047] In some exemplary embodiments, R₁₄ is chosen from hydrogen, optionally substituted (C₁-C₄)-alkyl, optionally substituted (C₁-C₄)-alkoxy, and optionally substituted (C₃-C₆)-cycloalkyl

[0048] In some exemplary embodiments R₈, R₉, R₁₂, and R₁₃ can be identical or different, and are chosen from hydrogen, methyl ethyl, and acetyl.

[0049] In some exemplary embodiments if the at least one molecule is chosen from formula (6), then R₁₂ and R₁₃ can be identical or different, and are chosen from



optionally substituted (C₁-C₂₀)-alkoxy, optionally substituted (C₃-C₈)-cycloalkyl, and optionally substituted (C₁-C₂₀)-alkyl, and R₁₄ is chosen from optionally substituted (C₁-C₂₀)-alkyl, optionally substituted (C₁-C₂₀)-alkoxy, and optionally substituted (C₃-C₈)-cycloalkyl.

[0050] In some exemplary embodiments, the at least one molecular entity is double stranded, the backbone of each strand may be the optionally same, and the backbone is polyamide backbone.

[0051] In some embodiments, in the compounds of Figure 1 and Figure 2, any of the R groups, R₁ - R₆ present are H, for example, R₂=H, R₃=H, R₄=H, R₅=H, and R₆=H, if they are present. In another embodiment, any of the R groups R₁ - R₆ present are methyl. In another embodiment, R₁ to R₆ are indenpently chosen from H and methyl. For example, some specific embodiments of bases of the invention are the structures shown in Figure 1 with the following substituents:

Base (Symbol)	R2	R3	R4	R5	R6
A	H or CH ₃	H	H		
isoA	H or CH ₃	H	H		
D	H or CH ₃	H			
G	H or CH ₃	H			
Gs	H or CH ₃	H			
I	H or CH ₃		H		
U				H or CH ₃	
U2s				H or CH ₃	
U4s				H or CH ₃	
C				H or CH ₃	H
Py-2o			H or CH ₃	H or CH ₃	
Cs				H or CH ₃	H
isoG	H or CH ₃	H			
isoGs	H or CH ₃	H			
Pu-2o	H or CH ₃		H		
isoC		H		H or CH ₃	
isoCs		H		H or CH ₃	
Py-4o			H or CH ₃	H or CH ₃	

[0052] In another embodiment, one or more of the H or CH₃ are independently substituted with a halogen such as Cl or F. (Note that Figures 1 and 2 and the above table use a different numbering of R groups than do the drawings above.)

[0053] Figure 7 illustrates other base pairing schemes compatible with the invention. Those involve Xanthine (X) or 2-thio-xanthine (2X) or 6-thio-xanthine (6X). X pairs with each of 2,4-diaminopyrimidine-5-yl, 2-aminopyrimidine-5-yl, and 4-aminopyrimidine-5-yl, while 2X also pairs with 2-aminopyrimidine-5-yl and 6X also pairs with 4-aminopyrimidine-5-yl. Accordingly, the choice of X or 2X or 6X in one strand opposed to the aminopyrimidine derivatives in the opposite strand allows one to control the level of degeneracy in a nucleic acid sequence. R₁ or "BB" in Figures 1, 2, or may be a point of attachment to a polymer backbone, for instance, a polyamide backbone.

Molecular Entities Defined from Base Pairs

[0054] In some embodiments, the bases and base-pairs are chosen from a sub-set of those depicted in Figures 1 and 2, and 7, depending on the needs of the application. In one embodiment, a composition comprising molecular entities, which are a pair of PNA oligomers which comprise a set of molecules comprising non-natural base purine-like molecules (such as formulas (1), (2), (3), (7), (9) and (10), and salts thereof) and pyrimidine-like molecules (such as formulas (4), (5), (6), (8), (11) and (12), and salts thereof).

[0055] In exemplary embodiments, the set of molecules is chosen from:

(a) at least one purine-like molecule of formula (1) or a salt thereof and at least one pyrimidine-like molecule of formula (5) or a salt thereof;

(b) at least one purine-like molecule of formula (2) or a salt thereof and at least one pyrimidine-like molecule of formula (6), or a salt thereof;

(c) at least one purine-like molecule of formula (3), or a salt thereof and at least one pyrimidine-like molecule of formula (8), or a salt thereof;

(d) at least one purine-like molecule of formula (7), or a salt thereof and at least one pyrimidine-like molecule of formula (4), or a salt thereof;

(e) at least one purine-like molecule of formula (9), or a salt thereof and at least one pyrimidine-like molecule of formula (12) or a salt thereof;

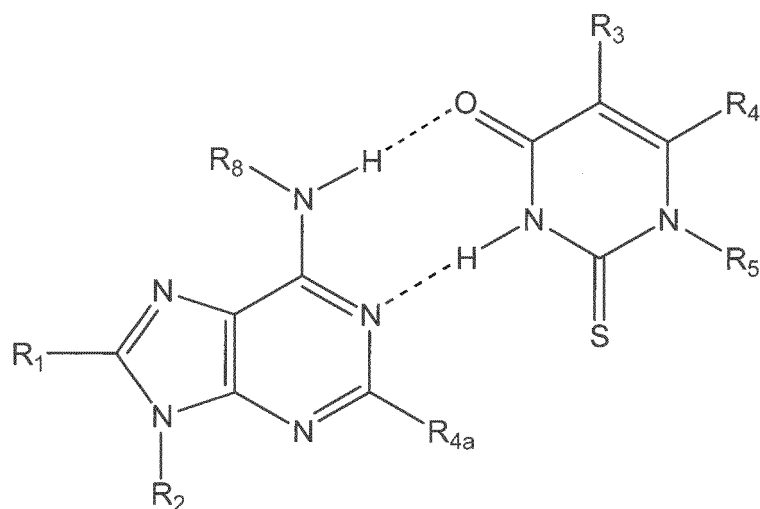
(f) at least one purine-like molecule of formula (10), or a salt thereof and at least one pyrimidine-like molecule of formula (11), or a salt thereof;

[0056] In some embodiments, the purine-like and pyrimidine-like molecules are chosen from a subset of the (a) through (f) pairings.

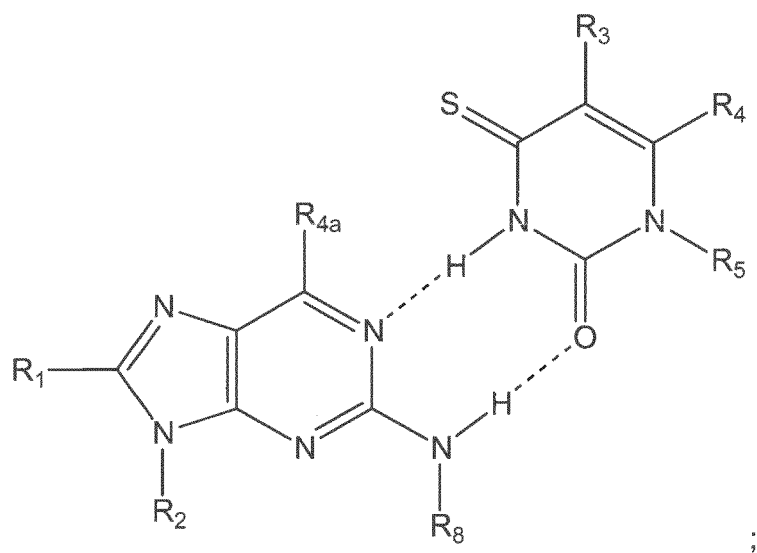
[0057] The specific choice of non-natural and natural base-pairs around which to build a sequence may depend on the needs of the application.

[0058] In one embodiment, the set of molecules is chosen from the following pairings::

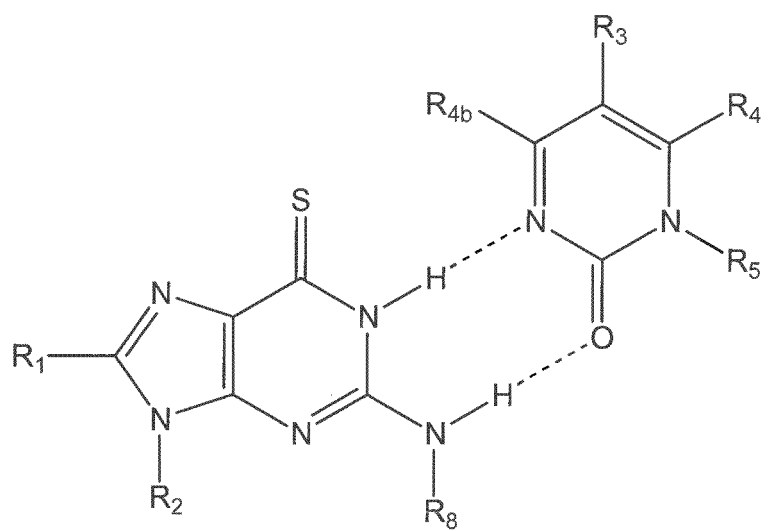
(a)



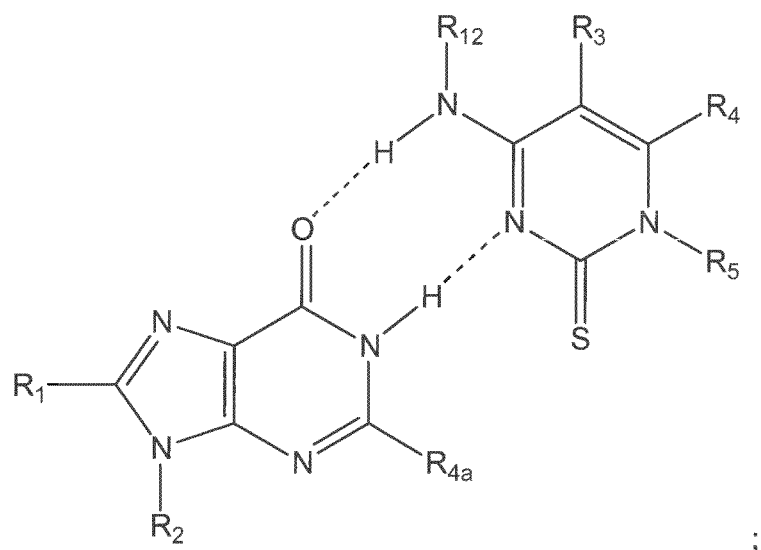
(b)



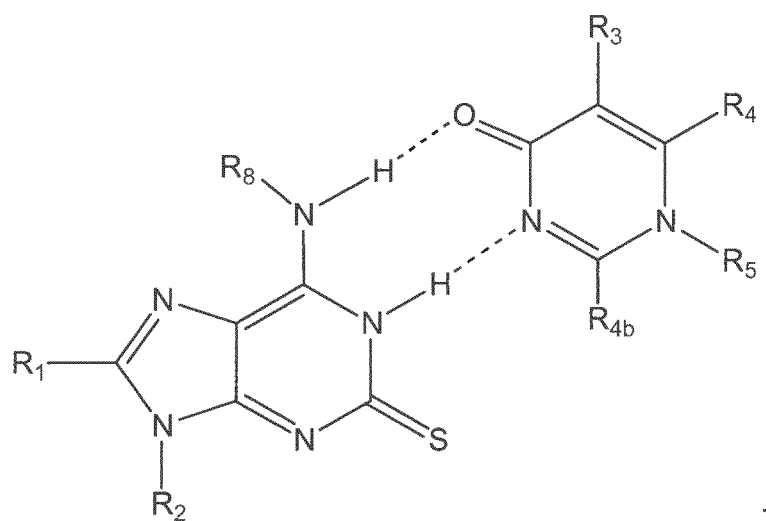
(c)



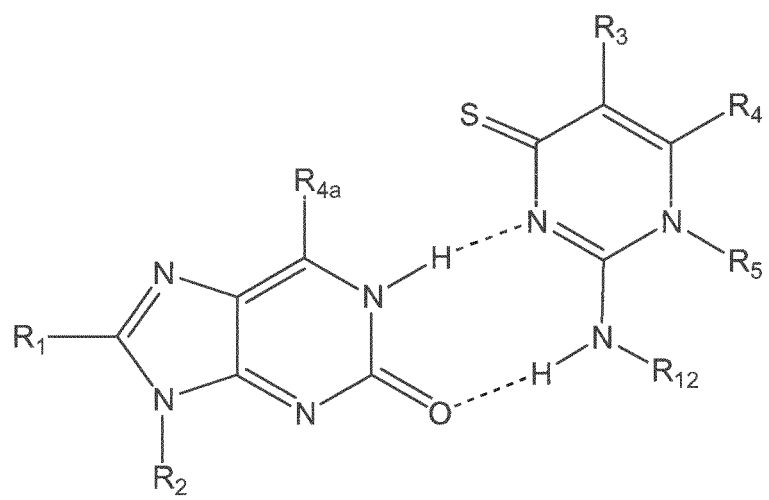
(d)



(e)



(f)



[0059] Examples of the pairing of certain base pairs comprised in the composition of claim 1 are shown in the following tables. The number of indicated hydrogen bonds is also given.

Table A

Base Pairs	formula (10)	formula (1)
formula (7)	3 H-bonds	2 H-bonds
formula (8)	repulsion	2 H-bonds

Table B

Base Pairs	formula (10)	formula (2)
formula (7)	3 H-bonds	2 H-bonds
formula (9)	repulsion	2 H-bonds

Table C

Base Pairs	formula (3)	formula (11)
formula (5)	3 H-bonds	2 H-bonds
formula (6)	repulsion	2 H-bonds

Table D

Base Pairs	formula (3)	formula (4)
formula (5)	3 H-bonds	repulsion
formula (12)	2 H-bonds	2 H-bonds

Table E

Base Pairs	formula (13)	formula (15)
formula (16)	3 H-bonds	2 H-bonds
formula (17)	repulsion	2 H-bonds

Table F

Base Pairs	formula (13)	formula (14)
formula (16)	3 H-bonds	repulsion
formula (18)	2 H-bonds	2 H-bonds

[0060] In one embodiment, a pair of molecular entities according to claim 1 may comprise the three hydrogen bond base pair IsoCytosine and isoGuanine. For example, the following are 2 hydrogen bond base pairs:

4-oxo-pyrimidine and isoGuanine

4-oxo-pyrimidine and isoThioGuanine

isoCytosine and 2-oxo-purine

isoThioCytosine and 2-oxo-purine

and the one hydrogen bond pair 4-oxo-purine and 2-oxo-purine. See Figure 2.

[0061] In another embodiment, a pair of molecular entities according to claim 1 may comprise the three hydrogen bond base pair 1,4-diaminopyrimidine-5-yl and Xanthine. Xanthine also may form two hydrogen bond base pairs with 1-diaminopyrimidine-5-yl or with 4-diaminopyrimidine-5-yl. In each of those pairings, one of the two amino groups from 1,4-diaminopyrimidine-5-yl is removed. See Figure 7, left side pairings. In yet another embodiment, 1 or 4-diaminopyrimidine-5-yl can form a two hydrogen bond base pair with a Xanthine derivative in which one of the two carbonyl oxygens is replaced by sulfur: 2-thioXanthine (X2s) or 6-thioXanthine (X6s). Mixing and matching these sorts of base pairings allows one to create a strand that is capable of hybridizing to more than one sequence on an opposite strand, thus allowing degeneracy in the binding of molecular entities carrying the different strands.

[0062] In one embodiment, the base pairs do not formally pair with any of the naturally occurring bases. In a further embodiment, the bases of the invention are used with the natural bases only, for example a single pair being sufficient to provide no DNA affinity and increased diversity.

Non-natural bases comprising halogen and alkyl groups

[0063] Other purine and pyrimidine-like molecules can also be used to vary affinity. For example, the number of hydrogen bonds may be varied by substituting the H for a halogen. In one embodiment, the bases of the invention are halogen-comprising non-natural bases, for example,

4-fluoro-2-oxo-pyrimidine (4-Ha-P)
 2-fluoro-Adenine (2-Ha-A)
 6-fluoro-isoAdenine (6-Ha-isoA)
 6-fluoro-Inosine (6-Ha-I)

[0064] Examples of base pairs include two hydrogen bonds

4-Ha-P : G
 4-Ha-P : Gs
 2-Ha-A : U
 2-Ha-A : U 2s
 6-Ha-isoA : U
 6-Ha-isoA : U4s
 6-Ha-I : C
 6-Ha-I : Cs

and one hydrogen bond pairs

4-Ha-P : I
 4-Ha-P : 6-Ha-I
 P : 6-Ha-I
 4-Ha-P : 6-Ha-I.

[0065] For example, fluorine and chlorine are quite small substituents that may have a relatively small steric effect on base pairing, i.e. 2-fluoroAdenine will pair with both uracil and 2-thioUracil, essentially acting like an analogue of Adenine. Sterically smaller alternatives, such as fluorine and chlorine, to an amino group can be used that face both a carbonyl and a thiocarbonyl. In one embodiment, larger halogens such as Iodine may be used to affect binding patterns, i.e. 2-Iodo-Adenine may pair with Uracil, but not with 2-thio-Uracil.

[0066] In another embodiment, 6-bromo-Adenine could pair with uracil via two hydrogen bond, such as A : U. Moreover, the binding to 2-thioUracil may be further enhanced by a favorable hydrophobic interactions between the bromine and the thiocarbonyl creating a pair of triple hydrogen bond strength similar to D : U.

[0067] In one embodiment, the bases of the invention are methyl analogues, i.e. Me for Ha or H. For example, 2-methyl-Adenine may be used as an Adenine analogue with PNAs. While it will strongly repel 2-thioUracil, it may bind to Uracil with significantly reduced affinity relative to Adenine.

[0068] In one embodiment, the above mentioned derivatives have hydrogen/halogen or an amino group in a potentially Watson-Crick like base pairing position facing either a carbonyl or thiocarbonyl on the "partner" base, i.e., the pattern determining the base pairing. Any other substitutions however is also possible, that does not directly influence the Watson-Crick like base pairing rules.

[0069] Non-limiting examples include: Pyrimidines substituted in the 5-position; i.e. bromouracil; analogues of C with one 4-N substituent, i.e. 4-N-alkyl and 4-N-acyl derivatives of C/C2s; purines halogenated in the 8-position, i.e. 8-bromo-

Adenine and 9-deaza and/or 8-aza purine derivatives; 2 and 6-amino"purines" with small N-substituents, i.e. N-6-Acetyl-diaminopurine, N-6-Metyl-Guanine, and N-Metyl-Adenine.

[0070] In one embodiment of the invention, the molecular entities have PNA backbones and are (PNA) oligomers.

Diversity of Base Pairs

[0071] One of skill in the art will appreciate that the increased number of bases will provide an increased degeneracy over natural bases. In one embodiment, the degeneracy minimizes secondary and tertiary structure. The degeneracy also may provide the advantage of one probe that can be used to recognize more than one target exclusively.

[0072] As described above, because of the increased diversity, within the same reading frame the system allows multiple different PNAs to be recognized with the same affinity by one other PNA complementary to all of the PNAs, yet also allow each PNA to be recognized with unique specificity by other complementary, yet different PNAs.

[0073] For example, using twelve bases, $12^{12} \sim 9 \times 10^{12}$ different 12'mers can be constructed. See Figure 6, for example. These can be divided into 91 subgroups, according to the number of complementary 12'mers with which they pair, from 1 to half a million. On average, each 12'mer will pair with 1400 others, creating 10^{16} different base pair combinations.

[0074] Thus, the information system of the invention is expended relative to "linear" information systems such as binary strings, bar codes, or natural oligomers based on A, C, G, U and T. In the present invention, what is read not only depends on the encoding entity, such as an oligomer, but also on which of the numerous complementary PNAs are selected to "read" it, i.e., bind or not bind.

[0075] The diversity of the system provides a degeneracy that limits cross reactivity. As one 12'mer will pair with 1400 others, for example, this limits the multiplexing capability of the system, i.e., only a limited number of pairs can work simultaneously and independently of each other with no cross reactivity. Triple vs. double vs. single hydrogen bonded base pairs are distinguished by different affinity.

[0076] For example, deaminated bases are expected to pair with approximately the same affinity with either carbonyl or thiocarbonyl bases (A:U = A:U2s; Aiso:U = Aiso:U4s; I:C = I:C_s; P:G = P:G_s). A single hydrogen bond pair, such as I:P, would similarly be expected to be weaker than I:C. This again provides even more diversity since for example, I and P each form tow stable two hydrogen bond complexes thus I will bind stronger to C and C_s than to P and P will bind stronger to G and G_s than to I.

[0077] Thus, for each large number of bases produced from a group of bases, many of the bases will be similar and provide a potential for cross hybridization. However, each pair of oligomers may also be quite unique with very different binding affinities.

[0078] The bases can thereby, for example, be used in a method to design a probe. Since, for example, each molecular entity such as a oligonucleotide made from a series of the bases of the invention, will bind to so many other molecular entities, a series of probes may be chosen or designed that differs slightly in the number of interactions, such as hydrogen bonds, that may be involved in the binding. Thus, for example, an oligonucleotide of 8 bases will have thousands of potential binding partners using the bases of the invention. Each of these may have slightly different strengths of interaction. Thus, one can chose a probe that is very sensitive to binding conditions, such as temperature, or design a series of probes that each bind at a slightly different temperature. The differences in temperature can be useful, in for example, immunohistochemistry in a system that can be varied by slight changes in temperature. The number of possible binding partners allows one to fine tune a binding partner for affinity, speed, temperature, low specific affinity for other probes or other compounds present in a sample and other uses.

[0079] A method of designing a probe may include a screening method where the thousands of possibilities for a certain length oligomer or numerous size oligomers are screened for the desired strength of hybridization and unique hybridization properties.

[0080] An entity may be chosen based on fast hybridization kinetics, combined with sufficient length to provide stability as links in supra molecular visualization and amplification systems and diagnostics.

[0081] For example, one may also use the system to design a series of probes or an information encoding system where each probe specifically hybridizes to a different target. One may further designs a specific detection system, in which one probe hybridizes to a group of or all of the probes (Figure 4). Such a system would be analogous to a hotel where each guest has a key unique to her own room, yet there is also a "master key" that will open every room.

[0082] The binding affinity between entities of base pairs may be a relative predictable function of length and triple, double, and single hydrogen bonded base pairs. The kinetic stability of the complexes once formed may follow a similar trend.

[0083] Binding affinity may be inversely proportional to the stability of the complex. For example, the longer and the more potential bond reach of an oligomer, the greater chance that it will be highly self structured and thus slow to hybridize. This can be expressed as a rate determining step preceding actual double-strand formation. More specifically, when two oligomers form a double-strand, the energy barrier to reaching the transition state will increase with the structure reorganization required of each of the hybridizing oligomers. Thus, one can reduce the energy barrier if the stable

structures of the oligomers before hybridization are aligned and/or by preparing oligomers that require little reorganization to reach the transition state because of lack of a stable self structure (Figure 5).

[0084] For example, intra molecular structure is a priori reduced by using more bases. For example, the 4 bases of DNA can form 16 pairs. Of these, 4 will be matches (25%) contributing on average with 2.5 hydrogen bonds. For example, 12 bases disclosed herein can form 144 pairs. Of these, only 22 will be matches (15%) and on average contribute with 2.1 hydrogen bonds. Since several consecutive base pairs are needed for stable intra molecular structures to form, this effect becomes quite pronounced. If it is assumed that 10 mutual hydrogen bonds are needed for a hair pin structure to form, and if 2 x 4 random DNA bases match, there is a 0.39% chance of hair pin formation. In contrast, using 12 bases instead of 4, there is only a 0.0084 % chance of a hair pin because 2 X 5 bases must match for it to form. Adding more bases also aids in smart design of a probe because one may avoid aggregates or crystalline structures.

[0085] In one embodiment, the bases of the invention may be used to prepare molecular entities such as PNA oligomers with at least one of high binding affinity, variable binding affinity, rapid binding kinetics and absence of interference with RNA/DNA.

[0086] The degeneracy of the bases may also be utilized to design a molecular encoding system similar to the natural information encoding system of DNA but greatly enhanced because of the greater number of bases.

[0087] The bases of the invention may be used in any application in which natural bases, DNA, PNA, etc, are used.

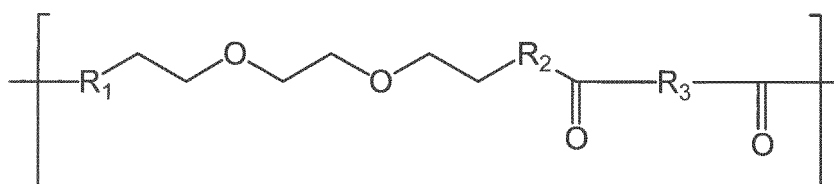
Linkers

[0088] In addition to connecting a base to the backbone, a linker may also be used to connect at least one molecular entity to another or to bind a molecular entity to another composition. A person of ordinary skill in the art of molecular conjugation knows numerous linkers. Examples include 6-amino-hexanoic acid, succinidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), homobifunctional linkers such as glutaric dialdehyde, hexane di-isocyanate, dimethylapimide, 1,5-difluoro-2,4-dinitrobenzene, divinyl sulfone (DVS), heterobifunctional linkers like e.g. N-gamma-maleimidobutyroxy succinimide ester (GMBS), and zero length linkers such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

[0089] Longer linker molecules based upon polyethylene glycol (PEG) are also available in the art. (See, for example, Discrete PEG (dPEG)[™] modification reagents available from Quanta Biodesign, Ltd., Powell, OH, or at www.quantabiodesign.com; PEG-based reagents available from EMD Biosciences, Inc., San Diego, CA, described in Novabiochem April, 2004, "Product focus: PEG reagents - bifunctional amino-PEG-acid spacers" brochure, available at www.novabiochem.com; and see Baumeister et al., Biopolymers, 71: 339 (2003); Kumar & Aldrich, Org. Lett., 5: 613 (2003). (See also, "Chemistry of Protein Conjugation and Cross-Linking" Shan S. Wong CRC Press, Boca Raton, Florida, USA, 1993; "BioConjugate Techniques" Greg T. Hermanson Academic Press, San Diego, California, USA, 1996; "Catalog of Polyethylene Glycol and Derivatives for Advanced PEGylation, 2004" Nektar Therapeutics Inc, Huntsville, Alabama, USA).

[0090] The present invention may also use a long uncharged linker comprising at least two units of the Formula I.

Formula I



[0091] In Formula I, R1 and R2 may comprise either NH or O, while R3 may comprise methyl, ethyl, propyl, CH₂-O-CH₂, and (CH₂-O-CH₂)₂. For example, in some embodiments of the instant invention, the linker comprises at least two units of the Formula I wherein R1 and R2 are both NH and R3 is CH₂-O-CH₂. See the examples that follow and the International Application entitled "MONOMERIC AND POLYMERIC

LINKERS USEFUL FOR CONJUGATING BIOLOGICAL MOLECULES AND OTHER

SUBSTANCES" for further description of this linker.

[0092] Fluoresceins may also be added to the linkers. When fluoresceins are spaced apart by an L30 oligomer larger

than L90 in an aqueous environment there may be negligible quenching. This contrasts with PEG-based spacers in aqueous solutions which may form a collapsed coiled structure.

[0093] For example, PNA probes with multiple fluorophores spaced via polymeric L30 may have higher water solubility, less aggregation and less non-specific binding, as compared to probes with just one fluorophore and no L30 spacer. Multiple PNAs joined together via L30 polymers may not aggregate and may have good solubility even with a total of 50-100 PNA bases in the same compound.

Detectable Labels

[0094] Compounds and compositions of the invention, including any molecular entity, may also include a detectable label which is any molecule which may be detected directly or indirectly so as to reveal the presence of a target in the sample. In some embodiments of the invention, a direct detectable label is used. Direct detectable labels may be detected per se without the need for additional molecules. Examples include fluorescent dyes, radioactive substances, and metal particles. In other embodiments of the invention, indirect detectable labels are used, which require the employment of one or more additional molecules. Examples include enzymes that affect a color change in a suitable substrate, as well as any molecule that may be specifically recognized by another substance carrying a label or react with a substance carrying a label. Other examples of indirect detectable labels thus include the non-natural bases of the present invention, antibodies, antigens, nucleic acids and nucleic acid analogs, ligands, substrates, and haptens.

[0095] Examples of detectable labels which may be used in the invention include fluorophores, chromophores, electrochemiluminescent labels, bioluminescent labels, polymers, polymer particles, bead or other solid surfaces, gold or other metal particles or heavy atoms, spin labels, radioisotopes, enzyme substrates, haptens, antigens, Quantum Dots, aminohexyl, pyrene, nucleic acids or nucleic acid analogs, or proteins, such as receptors, peptide ligands or substrates, enzymes, and antibodies (including antibody fragments).

[0096] Some detectable labels according to this invention comprise "color labels," in which the target is detected by the presence of a color, or a change in color in the sample. Examples of "color labels" are chromophores, fluorophores, chemiluminescent compounds, electrochemiluminescent labels, bioluminescent labels, and enzymes that catalyze a color change in a substrate. In some embodiments, more than one type of color may be used, for instance, by attaching distinguishable color labels to a single detection unit or by using more than one detection unit, each carrying a different and distinguishable color label.

[0097] "Fluorophores" as described herein are molecules that emit detectable electro-magnetic radiation upon excitation with electro-magnetic radiation at one or more wavelengths. A large variety of fluorophores are known in the art and are developed by chemists for use as detectable molecular labels and can be conjugated to the linkers of the present invention. Examples include fluorescein or its derivatives, such as fluorescein-5-isothiocyanate (FITC), 5-(and 6)-carboxyfluorescein, 5- or 6-carboxyfluorescein, 6-(fluorescein)-5-(and 6)-carboxamido hexanoic acid, fluorescein isothiocyanate, rhodamine or its derivatives such as tetramethylrhodamine and tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC). Other example fluorophores that could be conjugated to the instant linkers include: coumarin dyes such as (diethyl-amino)coumarin or 7-amino-4-methylcoumarin-3-acetic acid, succinimidyl ester (AMCA); sulforhodamine 101 sulfonyl chloride (TexasRedTM or TexasRedTM sulfonyl chloride; 5-(and-6)-carboxyrhodamine 101, succinimidyl ester, also known as 5-(and-6)-carboxy-X-rhodamine, succinimidyl ester (CXR); lissamine or lissamine derivatives such as lissamine rhodamine B sulfonyl Chloride (LisR); 5-(and-6)-carboxyfluorescein, succinimidyl ester (CFI); fluorescein-5-isothiocyanate (FITC); 7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester (DECCA); 5-(and-6)-carboxy-tetramethylrhodamine, succinimidyl ester (CTMR); 7-hydroxycoumarin-3-carboxylic acid, succinimidyl ester (HCCA); 6->fluorescein-5-(and-6)-carboxamido!hexanoic acid (FCHA); N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacenepropionic acid, succinimidyl ester; also known as 5,7-dimethylBODIPYTM propionic acid, succinimidyl ester (DMBP); "activated fluorescein derivative" (FAP), available from Molecular Probes, Inc.; eosin-5-isothiocyanate (EITC); erythrosin-5-isothiocyanate (ErITC); and CascadeTM Blue acetylazide (CBAA) (the O-acetylazide derivative of 1-hydroxy-3,6,8-pyrenetrisulfonic acid). Yet other potential fluorophores useful in this invention include fluorescent proteins such as green fluorescent protein and its analogs or derivatives, fluorescent amino acids such as tyrosine and tryptophan and their analogs, fluorescent nucleosides, and other fluorescent molecules such as Cy2, Cy3, Cy 3.5, Cy5, Cy5.5, Cy 7, IR dyes, Dyomics dyes, phycoerythrin, Oregon green 488, pacific blue, rhodamine green, and Alexa dyes. Yet other examples of fluorescent labels which may be used in the invention include and conjugates of R-phycoerythrin or allo-phycoerythrin, inorganic fluorescent labels such as particles based on semiconductor material like coated CdSe nanocrystallites.

[0098] A number of the fluorophores above, as well as others, are available commercially, from companies such as Molecular Probes, Inc. (Eugene, OR), Pierce Chemical Co. (Rockford, IL), or Sigma-Aldrich Co. (St. Louis, MO).

[0099] Examples of polymer particles labels which may be used in the invention include micro particles, beads, or latex particles of polystyrene, PMMA or silica, which can be embedded with fluorescent dyes, or polymer micelles or capsules which contain dyes, enzymes or substrates.

[0100] Examples of metal particles which may be used in the invention include gold particles and coated gold particles, which can be converted by silver stains.

[0101] Examples of haptens that may be conjugated in some embodiments are fluorophores, myc, nitrotyrosine, biotin, avidin, streptavidin, 2,4-dinitrophenyl, digoxigenin, bromodeoxy uridine, sulfonate, acetylaminofluorene, mercury trinitrophenol, and estradiol.

[0102] Examples of enzymes which may be used in the invention comprise horse radish peroxidase (HRP), alkaline phosphatase (AP), beta-galactosidase (GAL), glucose-6-phosphate dehydrogenase, beta-N-acetylglucosaminidase, β -glucuronidase, invertase, Xanthine Oxidase, firefly luciferase and glucose oxidase (GO).

[0103] Examples of commonly used substrates for horse radish peroxidase (HRP) include 3,3'-diaminobenzidine (DAB), diaminobenzidine with nickel enhancement, 3-amino-9-ethylcarbazole (AEC), Benzidine dihydrochloride (BDHC), Hanks-Yates reagent (HYR), Indophane blue (IB), tetramethylbenzidine (TMB), 4-chloro-1-naphthol (CN), α -naphthol pyronin (α -NP), o-dianisidine (OD), 5-bromo-4-chloro-3-indolylphosphate (BCIP), Nitro blue tetrazolium (NBT), 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT), tetranitro blue tetrazolium (TNBT), 5-bromo-4-chloro-3-indoxyl-beta-D-galactoside/ferro-ferricyanide (BCIG/FF).

[0104] Examples of commonly used substrates for Alkaline Phosphatase include Naphthol-AS-B1-phosphate/fast red TR (NABP/FR), Naphthol-AS-MX-phosphate/fast red TR (NAMP/FR), Naphthol-AS-B1-phosphate/fast red TR (NABP/FR), Naphthol-AS-MX-phosphate/fast red TR (NAMP/FR), Naphthol-AS-B1-phosphate/new fuchsin (NABP/NF), bromochloroindolyl phosphate/nitroblue tetrazolium (BCIP/NBT), 5-Bromo-4-chloro-3-indolyl-b(beta)-d(delta)-galactopyranoside (BCIG).

[0105] Examples of luminescent labels which may be used in the invention include luminol, isoluminol, acridinium esters, 1,2-dioxetanes and pyridopyridazines. Examples of electrochemiluminescent labels include ruthenium derivatives.

[0106] Examples of radioactive labels which may be used in the invention include radioactive isotopes of iodide, cobalt, selenium, hydrogen, carbon, sulfur and phosphorous.

[0107] In some embodiments the detection unit may comprise from 1 up to 500 detectable label molecules. In some embodiments, the detectable label is an enzyme, which may be conjugated to a polymer, such that the number of enzyme molecules conjugated to each polymer molecule is, for instance, 1 to 200, 2 to 50, or 2 to 25. In some embodiments, the detectable label is a gold particle, a radioactive isotope, or a color label, e.g. a low molecular weight fluorochrome, and the number of detectable labels conjugated to each polymer molecule is, for instance, 1 to 500, or for instance, 2 to 200. In some embodiments, the detectable label is a protein fluorochrome and the number of detectable labels conjugated to each polymer molecule is 1-50, 2-20. In some embodiments, the number of detectable label molecules conjugated to each polymer is 1-200, 2-50, 2-25, or is 10-20, 5-10, or 1-5. Add some more number ranges to pick from later on if necessary.

[0108] The detectable label can be detected by numerous methods, including, for example, reflectance, transmittance, light scatter, optical rotation, and fluorescence or combinations hereof in the case of optical labels or by film, scintillation counting, or phosphorimaging in the case of radioactive labels. See, e.g., Larsson, 1988, Immunocytochemistry: Theory and Practice, (CRC Press, Boca Raton, FL); Methods in Molecular Biology, vol. 80 1998, John D. Pound (ed.) (Humana Press, Totowa, NJ). In some embodiments, more than one detectable label is employed.

[0109] When more than one color label is used, the different colors may have different, distinguishable colors. In some embodiments both colors can be detected simultaneously, such as by fusion or juxtaposition of the signals, signal enhancement or quenching, or detection of multiple colors in the sample. The exact choice of detectable label or combinations of detectable labels may be based on personal preferences in combinations with restrictions of the sample type, sample preparation method, detection method and equipment, and optional contrasting labels used in the sample.

Hybridization of Base Pairs

[0110] Two different base pairs or molecular entities may specifically hybridize. In some embodiments, the chosen hybridization conditions are "stringent conditions," meaning herein conditions for hybridization and washes under which nucleotide sequences that are significantly complementary to each other remain bound to each other. The conditions are such that sequences at least 70%, at least 80%, at least 85-90% complementary remain bound to each other. The percent complementary is determined as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389-3402.

[0111] Specified conditions of stringency are known in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ausubel et al. 1995 eds.), sections 2, 4, and 6 (hereby incorporated by reference). Additionally, specified stringent conditions are described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Press, chapters 7, 9, and 11.

[0112] In other embodiments, the chosen hybridization conditions are "high stringency conditions." An example of high stringency hybridization conditions is hybridization in 4X sodium chloride/sodium citrate (SSC) at 65-70°C or hybridization in 4X SSC plus 50% formamide at 42-50°C, followed by one or more washes in 1X SSC, at 65-70°C. It will be understood

that additional reagents may be added to hybridization and/or wash buffers, e.g., blocking agents (BSA or salmon sperm DNA), detergents (SDS), chelating agents (EDTA), Ficoll, PVP, etc.

[0113] In yet other embodiments, the chosen conditions are "moderately stringent conditions." Moderate stringency, as used herein, includes conditions that can be readily determined by those having ordinary skill in the art based on, for example, the length of the molecular entity or a specific nucleic acid or nucleic acid analog segment. Exemplified conditions are set forth by Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2d ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press (1989), and include use of a prewashing solution of 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization conditions of 50% formamide, 6X SSC at 42°C (or other similar hybridization solution, such as Stark's solution, in 50% formamide at 42°C), and washing conditions of 60°C, 0.5X SSC, 0.1 % SDS.

[0114] In some embodiments, the chosen conditions are "low stringency" conditions. Low stringency conditions may include, as used herein, conditions that can be readily determined by those having ordinary skill in the art based on, for example, the length of the molecular entity. Low stringency may include, for example, pretreating the segment for 6 hours at 40°C in a solution containing 35% formamide, 5 x SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% W/V dextran sulfate, and 5-20x10⁶ CPM probe is used. Samples are incubated in hybridization mixture for 18-20 hours at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2 x SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1 % SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C.

Polymers

[0115] The compounds and compositions of the invention, including any molecular entity may also comprise at least one polymer. A "polymer," as used herein, may be any molecule that facilitates covalent or non-covalent attachment of one or more other components or molecular entities. For instance, the polymer may facilitate the attachment of one or more probes, molecular entities, nucleic acid analog segments, and/or detectable labels. The polymer may be a soluble molecule or an insoluble molecule and may have any shape including a linear polymer, branched polymer, bead or other globular shaped polymer.

[0116] Examples of suitable polymers include polysaccharides such as dextrans, carboxy methyl dextran, dextran polyaldehyde, carboxymethyl dextran lactone, and cyclodextrins; pullulans, schizophyllan, scleroglucan, xanthan, gellan, O-ethylamino guaran, chitins and chitosans such as 6-O-carboxymethyl chitin and N-carboxymethyl chitosan; derivatized celluloses such as carboxymethyl cellulose, carboxymethyl hydroxyethyl cellulose, hydroxyethyl cellulose, 6-amino-6-deoxy cellulose and O-ethylamine cellulose; hydroxylated starch, hydroxypropyl starch, hydroxyethyl starch, carrageenans, alginates, and agarose; synthetic polysaccharides such as ficoll and carboxymethylated ficoll; vinyl polymers including poly(acrylic acid), poly(acryl amides), poly(acrylic esters), poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(maleic acid), poly(maleic anhydride), poly(acrylamide), poly(ethyl-co-vinyl acetate), poly(methacrylic acid), poly(vinyl alcohol), poly(vinyl alcohol-co-vinyl chloroacetate), aminated poly(vinyl alcohol), and co block polymers thereof; poly ethylene glycol (PEG) or polypropylene glycol or poly(ethylene oxide-co-propylene oxides) containing polymer backbones including linear, comb-shaped or hyperbranched polymers and dendrimers, including branched PAMAM-dendrimers; poly amino acids including polylysines, polyglutamic acid, polyurethanes, poly(ethylene imines), pluriol; proteins including albumins, immunoglobulins, and virus-like proteins (VLP), and polynucleotides, DNA, PNA, LNA, oligonucleotides and oligonucleotide dendrimer constructs. Also contemplated is the use of mixed polymers, i.e., a polymer comprised of one or more of the above examples including any of the polymers, the co-block polymers and random co-polymers.

[0117] Properties of the polymer can be varied, depending on the desired application, to optimize performance. Examples of parameters that may be considered in the choice of a polymer include the length of the polymer and branching of the polymer. Furthermore, the polymer may carry various substituents. The substituents may be chemically protected and/or activated, allowing the polymer to be derivatized further.

Kits

[0118] Compositions according to the invention may be comprised in a kit. The kit may optionally comprise one or more binding agents, and suitable reagents for, for instance: antigen retrieval, sample dilution, reagent dilution, blocking of non-specific binding, blocking of endogenous enzyme activity, or blocking of repetitive sequences. The kit may optionally also comprise at least one container, instructions for use, and reference targets or samples.

EXAMPLES**Example 1a. Preparation of Pyrimidinone-monomer****[0119]**

1. In dry equipment 4.6 g of solid Na in small pieces was added to 400 mL ethanol (99.9%), and was dissolved by stirring. Hydroxypyrimidine hydrochloride, 13.2 g, was added and the mixture refluxed for 10 minutes. Then 12.2 mL ethyl-bromoacetate (98%) was added and the mixture refluxed for 1½ hour. The reaction was followed using Thin Layer Chromatography (TLC). The ethanol was evaporated leaving a white compound, which was dissolved in a mixture of 80 mL of 1 M NaCitrate (pH 4.5) and 40 mL of 2M NaOH. This solution was extracted four times with 100 mL Dichloromethane (DCM). The DCM phases were pooled and washed with 10 mL NaCitrate/NaOH - mixture. The washed DCM phases were evaporated under reduced pressure and resulted in 17.2 g of crude solid product. This crude solid product was recrystallized with ethylacetate giving a yellow powder. The yield for this step was 11.45 g (63 %).

2. The yellow powder, 12.45 g. from above was hydrolyzed by refluxing overnight in a mixture of 36 mL DIPEA, 72 mL water and 72 mL dioxane. The solvent was evaporated and water was removed from the residue by evaporation from toluene. The yield for this step was 100%.

3. OBS. Pyrimidinone acetic acid (10.5 g), 16.8 g PNA-backbone ethylester, 12.3 g DHBT-OH, 19 mL Triethylamine was dissolved in 50 mL N,N-dimethylformamide (DMF). DIPIDIC (11.8 mL) was added and the mixture stirred overnight at room temperature. The product was taken up in 100 mL DCM and extracted three times with 100 mL of dilute aqueous NaHCO₃. The organic phase was extracted twice with a mixture of 80 mL of 1 M NaCitrate and 20 mL of 4M HCl. Because TLC showed that some material was in the citrate phase, it was extracted twice with DCM. The organic phases were pooled and evaporated. Because there was a precipitation of urea, the product was dissolved in a DCM, and the urea filtered off. Subsequent evaporation left an orange oil. Purification of the orange oil was performed on a silica column with 10% methanol in DCM. The fractions were collected and evaporated giving a yellow foam. The yield for this step was 7.0 g (26.8 %).

4. The yellow foam (8.0 g) was hydrolyzed by reflux overnight in 11 mL DIPEA, 22 mL water, and 22 mL dioxane. The solvent was evaporated and the oil was dehydrated by evaporation from toluene leaving an orange foam. The yield for this step was 100 %.

Example 1 b. Alternative method of preparing pyrimidinone monomer

[0120] Step 1. In dry equipment 9.2 g of solid Na in small pieces was dissolved in 400 mL ethanol (99.9%), with stirring. Hydroxypyrimidine hydrochlorid, 26.5 g, was added, and the mixture was stirred for 10 minutes at 50°C. Then 24.4 mL Ethyl bromoacetate (98%) was added and the mixture stirred at 50°C for 1 hour. The reaction was followed using Thin Layer Chromatography (TLC).

[0121] The ethanol was evaporated leaving a white compound, which was dissolved in 70 mL of water and extracted with 20 mL DCM. Another 30 mL of water was added to the water phase, which was extracted with 3x100 mL DCM. The DCM-phase from the first extraction contains a lot of product, but also some impurities, wherefore this phase was extracted twice with water. These two water phases then were back extracted with DCM.

[0122] The combined DCM phases were pooled and washed with 10 mL water. The washed DCM phases were evaporation under reduced pressure and resulted in 25.1 g yellow powder. The yield for this step was 25.1 g = 69 %. Maldi-Tof: 181.7 (calc. 182).

[0123] Step 2. 34.86 g yellow powder from above was dissolved in 144 mL 2M NaOH. After stirring 10 minutes at room temperature, the mixture was cooled in an ice bath. Now 72 mL 4 M HCl (cold) was added. The product precipitated. After stirring for 5 minutes, the precipitate was filtered and thoroughly washed with ice water. Drying in a dessicator under reduced pressure left 18.98 g yellow powder. The yield for this step was 18.98 g = 64 %.

[0124] Step 3. Pyrimidinone acetic acid 11.1 g and triethylamine 12.5 mL were dissolved in N,N-dimethylformamide (DMF) 24 ml, HBTU 26.2 g was added plus 6 mL extra DMF. After 2 minutes a solution of PNA-Backbone ethylester 14.7g dissolved in 15 mL DMF was added. The reaction mixture was stirred at room temperature and followed using TLC. After 1½hour precipitate had formed. This was filtered off.

[0125] The product was taken up in 100 mL DCM and extracted with 2 x 100 mL dilute aqueous NaHCO₃. Both of the aqueous phases were washed with a little DCM. The organic phases were pooled and evaporated. Evaporation left an orange oil. Purification of the product was done on a silica column with 10-20% methanol in ethylacetate. The fractions were collected and evaporated giving a yellow oil. The oil was dissolved and evaporated twice from ethanol. The yield from this step was 20.68 g = 90 %.

[0126] Step 4. The yellow oil (18.75 g) was dissolved in 368 mL 0.2 M Ba(OH)₂. Stirring for 10 minutes before 333

mL 0.221 M H₂SO₄ was added. A precipitation was performed immediately. Filtration through cellite, which was washed with water. The solvent was evaporated. Before the evaporation was at end, the product was centrifuged to get rid of the very rest of the precipitation. Re-evaporation of the solvent left a yellow oil. The yield from this step was 13.56 g = 78 %.

[0127] Step 5. To make a test on the P-monomer 3 consecutive P's were coupled to Boc-L300-Lys(Fmoc) -resin, following normal PNA standard procedure. The product was cleaved from the resin and precipitated also following standard procedures: HPPP-L300-Lys(Fmoc). Maldi-Tof on the crude product: 6000 (calc. 6000) showing only minor impurities.

Example 2. Preparation of thio-guanine monomer

[0128]

1. 6-Chloroguanine (4.93 g) and 10.05 g K₂CO₃ was stirred with 40 mL DMF for 10 minutes at room temperature. The reaction mixture was placed in a water bath at room temperature and 3.55 mL ethyl bromoacetate was added. The mixture was stirred in a water bath until TLC (20% Methanol/DCM) showed that the reaction was finished. The precipitated carbonate was filtered off and washed twice with 10 mL DMF. The solution, which was a little cloudy, was added to 300 ml water, whereby it became clear. On an ice bath the target compound slowly precipitated. After filtration the crystals were washed with cold ethanol and dried in a desiccator. The yield for this step was 3.3 g (44.3 %) of ethyl chloroguanine acetate.

2. Ethyl chloroguanine acetate (3.3 g) was dissolved by reflux in 50 mL absolute ethanol. Thiourea (1.08 g) was added. After a refluxing for a short time, precipitate slowly began forming. According to TLC (20% Methanol/DCM) the reaction was finished in 45 minutes. Upon completion, the mixture was cooled on an ice bath. The precipitate was then filtered and dried overnight in a desiccator. The yield for this step was 2.0 g (60 %) ethyl thioguanine acetate.

3. Ethyl thioguanine acetate (3.57 g) was dissolved in 42 mL DMF. Benzylbromide (2.46 mL) was then added and the mixture stirred in an oil bath at 45°C. The reaction was followed using TLC (25% Methanol/DCM). After 3 hours all basis material was consumed. The step 3 target compound precipitated upon evaporation under reduced pressure and high temperature. The precipitate was recrystallized in absolute ethanol, filtered and then dried in a desiccator. The yield for this step was 3.88 g (82 %) of methyl benzyl thioguanine ethylester.

4. Methyl benzyl thioguanine ethylester (5.68 g) was dissolved in 12.4 mL of 2M NaOH and 40 mL THF, and then stirred for 10 minutes. The THF was evaporated by. This was repeated. The material was dissolved in water and then 6.2 mL of 4M HCl was added, whereby the target product precipitated. Filtering and drying in a desiccator. The yield for this step was 4.02 g (77 %).

5. The product of step 4 (4.02 g), 3.45 g backboneethylester, 9 mL DMF, 3 mL pyridine, 2.1 mL triethylamine and 7.28 g PyBop were mixed and then stirred at room temperature. After 90 minutes a solid precipitation formed. The product was taken up in 125 mL DCM and 25 mL methanol. This solution was then extracted, first with a mixture of 80 mL of 1 M NaCitrate and 20 mL of 4M HCl, and then with 100 mL dilute aqueous NaHCO₃. Evaporation of the organic phase gave a solid material. The material was dissolved in 175 mL boiling ethanol. The volume of the solution was reduced to about 100 mL by boiling. Upon cooling in an ice bath, the target product precipitate. The crystals were filtered, washed with cold ethanol and then dried in a desiccator. The yield of this step was 6.0 g (86 %).

6. The product of step 5 (6.0 g) was dissolved in 80 mL THF, 7.5 mL 2M NaOH and 25 mL water. The solution became clear after ten minutes of stirring. THF was evaporated. Water (50 mL) was added to the mixture. THF was evaporated. Water (50 mL) was added to the mixture. When the pH was adjusted by the addition of 3.75 mL of 4M HCl, thio-guanine monomer precipitated. It was then filtered, washed with water and dried in a desiccator. The yield for this step was 5.15 g (91 %).

Example 3. Preparation of Diaminopurine acetic acid ethyl ester

[0129] 1. Diaminopurine (10 g) and 40 g of K₂CO₃ were added to 85 mL of DMF and stirred for 30 minutes. The mixture was cooled in a water bath to 15°C. Ethyl bromoacetate (3 mL) was added three times with 20 minute intervals between each addition. This mixture was then stirred for 20 minutes at 15°C. The mixture was left in the water bath for another 75 minutes, and the temperature increased to 18°C. The DMF was removed by filtering and the remaining K₂CO₃ was added to 100 mL of ethanol and refluxed for 5 minutes. Filtering and repeated reflux of the K₂CO₃ in 50 mL ethanol, filtering. The pooled ethanol phases were placed in a freezer, after which crystals formed. These crystals were filtered, washed with cold ethanol, filtered again and then dried in a desiccator overnight. The yield for this step was 12 g (76 %).

Example 4. Preparation of L₃₀-Linker

[0130]

1. A solution of 146 mL of 2,2'-(Ethylenedioxy)bis(ethylamine) (98%) in 360 mL of THF was cooled in an ice bath. Di-tert-butyl dicarbonate (97%) (65 g) in 260 mL THF was added dropwise over one hour. The solvent was evaporated. The remaining oil was dissolved in water and then evaporated off. The oily product was dissolved in 300 mL water, extracted with 300 mL DCM, then washed twice with 150 mL of DCM. The collected organic phase was washed with 50 mL of water before evaporating to about half the volume. The organic phase was then extracted with 400 mL of 1 M NaCitrate (pH 4.5), and then extracted again with 50 mL of 1 M NaCitrate (pH 4.5). The aqueous phases were washed with 50 mL DCM before cooling on an ice bath. While stirring, 100 mL of 10M NaOH was added to the aqueous washed aqueous phases resulting in pH of 13-14. In a separation funnel the product separated on its own. It was shaken with 300 mL DCM and 50 ml water. The organic phase was evaporated, yielding a white oil. The yield for this step was 48.9 g (65.7 %). The product had a predicted molecular formula of $C_{11}H_{24}N_2O_4$ (MW 248.3).

2. Boc-amine (76.2 g) was dissolved in 155 mL pyridine. Diglycolic anhydride (54.0 g) (90%) was added. After stirring for 15 minutes the intermediate product separated out and then 117 mL Acetic Anhydride (min. 98%) was added and the mixture stirred at 95°C for 1 hour. The solution was then put under reduced pressure and evaporated. Water (117 mL) was added, and the mixture was then stirred for 15 minutes, after which 272 mL of water and 193 mL of DCM were added. The organic layer was extracted twice with 193 mL of 1 M Na_2CO_3 and then twice with a mixture of 72 mL of 4M HCl and 289 mL of 1 M NaCitrate. After each extraction the aqueous phase was washed with a little DCM. The collected organic phase was washed with 150 mL of water. The solvent was evaporated leaving the product as an orange oil. This yield for this step was 100.3 g (0.29 mol) (94 %). The product had a predicted molecular formula of $C_{15}H_{26}N_2O_7$ (MW 346.4).

3. The product from step 2 (100.3 g) was dissolved in an equal amount of THF and was then added dropwise to 169.4 mL of 2,2'-(Ethylenedioxy)bis(ethylamine) at 60°C over the period of 1 hour. The amine was distilled from the reaction mixture at 75-80° C and a pressure of 3×10^{-1} mBar. The residue from the distillation was taken up in a mixture of 88 mL of 4M HCl and 350mL of 1 M NaCitrate and then extracted three times with 175 mL of DCM. The aqueous phase was cooled in an ice bath and was cautiously added to 105 mL of 10M NaOH while stirring. In a separation funnel the product slowly separated from the solution. When separated 100 mL of water and 950 mL of DCM were added to the product. Stirring for some minutes before pouring to a separation funnel. The pH in the aqueous phase should be 14. The aqueous phase was extracted four times with 150 mL of DCM. The solvent was evaporated. The oily residue was dehydrated by evaporation from toluene, giving a yellow oil. The yield for this step was 115.48 g (81 %). The product had a predicted molecular formula of $C_{21}H_{42}N_4O_9$ (MW 494.6).

4. The Boc-amine (115.48 g) from step 3 was dissolved in 115 mL of pyridine. Diglycolic anhydride (40.6 g) (90%) was added and the mixture stirred for 15 minutes, after which the intermediate product came out. Acetic Anhydride (97 mL) (min. 98%) was added and the mixture stirred at 95°C for 1 hour. The mixture was then evaporated under reduced pressure. The mixture was then cooled and then 80 mL of water was added. This mixture was stirred for 15 minutes and then 200 mL of water and 150 mL of DCM were added. The organic layer was extracted twice with 150 mL of 1 M Na_2CO_3 and then twice with a mixture of 53 mL of 4M HCl and 213 mL of 1 M NaCitrate. After each extraction the aqueous phase was washed with a little DCM. The collected organic phase was washed with 150 mL of water. The solvent was evaporated. The oily residue was dehydrated by evaporation from toluene, giving a yellow oil. The yield for this step was 125 g (92 %). The product had a predicted molecular formula of $C_{25}H_{44}N_4O_{12}$ (MW 592.6), with a mass spectrometry determined molecular weight of 492.5.

Further purifying of the product could be done on a silica column with a gradient from 5-10% methanol in DCM. The yield from the column purification was 69% and produced a white oil.

5. White oil (12.4 g) from step 4 was dissolved in a mixture of 12 mL water and 12 mL 1,4-Dioxane (99 %) and was then heated to reflux. DIPEA (6 mL) was added and refluxed for 30 minutes. This mixture was cooled and then evaporated. The oily residue was dehydrated by evaporation from toluene, giving a yellow oil. The product had a predicted molecular formula of $C_{25}H_{46}N_4O_{14}$ (MW 610.6).

Example 5. Exemplary embodiments of PNA sequences

[0131] All made by PNA standard procedures (see Example 18)

Table 1.

Sequence designation	PNA sequences ¹	N-terminal	C-terminal	Molecular Weight
Seq. AA	TCD-DG _s G _s -TAC-A	Flu-L ₃₀ -	-Lys(Cys)	8805
Seq. AB	U _s GU _s -DPP-TTG-D	Flu-L ₃₀ -	-Lys(Cys)	8727
Seq. AC	CU _s G _s -G _s DD-TU _s D-G _s DC	Flu-L ₃₀ -	-Lys(Cys)	9413

(continued)

Sequence designation	PNA sequences ¹	N-terminal	C-terminal	Molecular Weight
Seq. AD	GTP-TAA-TTP-PAG	Flu-L ₃₀ -	-Lys(Cys)	9203
Seq. AE	DG _s T-CG _s D-DG _s G-U _s CU _s	Flu-L ₃₀ -	-Lys(Cys)	9413
Seq. AF	AGA-CPT-TPG-APT	Flu-L ₃₀ -	-Lys(Cys)	9187
Seq. AG	TCD-DII-TAC-A	Flu-L ₃₀ -	-Lys(Cys)	8742
¹ Flu is fluorescein; T is thiamine; C is cytosine; D is diaminopurine; G _s is thioguanine; A is Adenine; U _s is 2/4-thiouracil; G is guanine; P is pyrimidone; I is inosine.				

Example 6 - Three PNAs with the L₃₀ linker with different amino acids at the C-terminal**[0132]**BA: Flu-L₃₀-DGT-DTC-GTD-CCG-Lys(Acetyl)BB: Flu-L₃₀-DGT-DTC-GTD-CCG-Lys(Cys)BC: Flu-L₃₀-DGT-DTC-GTD-CCG-Lys(Lys)₃**Example 7 - Synthesis of Flu-L₉₀-Lys(Flu)-L₃₀-Lys(Cys)**

[0133] Using procedure provided in Example 18a, an MBHA-resin was loaded with Boc-Lys(Dde)-OH. Using a peptide synthesizer, amino acids were coupled according to PNA solid phase procedure provided in Example 18d yielding Boc-L₉₀-Lys(Fmoc)-L₃₀-Lys(Dde). The Boc and Fmoc protections groups were removed and the amino groups marked with fluorescein using the procedure in Example 18e. Then, the Dde protection group was removed and 0.4 M cysteine was added according to the procedure in Example 18b. The PNA was cleaved from the resin, precipitated with ether and purified on HPLC according to Example 18d. The product was found to have a molecular weight of 3062 using MALDI-TOF mass spectrometry; the calculated molecular weight is 3061.

Example 8 - Synthesis of a conjugate made from sequence AA from Example 5, DexVS70, and Flu(10)

[0134] Dextran (with a molecular weight of 70 kDa) was activated with divinylsulfone to a degree of 92 reactive groups/dextran polymer; this product is designated DexVS70.

280 μ L DexVS70	20 nmol
66 μ L Flu ₂ Cys	160 nmol (prepared from Example 7)
25 μ L 0.8 M NaHCO ₃ pH=9.5	
29 μ L H ₂ O	

[0135] The above four compounds were mixed. The mixture was placed in a water bath at 30° C for 16 hours. The mixture was added to 50 nmol of freeze-dried PNA (sequence AA from Example 5). The mixture was placed in a water bath at 30° C for 30 minutes. The conjugating reaction was quenched with 50 μ L of 500 mM cysteine for 30 minutes at 30° C. Purification of the product was performed using FPLC: column SUPERDEX®- 200, buffer 10 mM Hepes 100 mM NaCl, method 7 bank 2, Loop 1 mL. Two fractions were collected: one with the product and one with the residue. The relative absorbance Flu₂ (ϵ_{500nm} = 146000 M⁻¹, ϵ_{260nm} = 43350 M⁻¹) and PNA (ϵ_{500nm} = 73000 M⁻¹, ϵ_{260nm} = 104000 M⁻¹) was used to calculate the average conjugation ratio of Flu₂, PNA, and DexVS70. The conjugation ratio of Flu₂ to DexVS70 was 9.4. The conjugation ratio of PNA (sequence AA) to DexVS70 was 1.2.

Example 9. Synthesis of HRP-DexVS70-Seq. AA

[0136] Using the procedure of Example 14, the conjugate HRP-DexVS70-Seq. AA was made. The ratio of HRP to DexVS70 is 12.2; the ratio of Seq. AA to Dex70 is 1.2.

Example 10. Synthesis of GaM-DexVS70-Seq. AB

[0137] The synthesis of GaM-DexVS70-Seq. AB was performed using the procedure in Example 16 with the following changes as indicated.

[0138] Dextran (molecular weight 70 kDa) is activated with divinylsulfone to a degree of 92 reactive groups/dextran polymer.

105.0 μ L DexVS70 7.5 nmol
 57.0 μ L Goat anti mouse IgG (GAM-Ig) 15 nmol
 8.9 μ L 4 M NaCl
 10.6 μ L 0.8 M NaHCO₃ (pH = 9.5)
 144.5 μ L H₂O

[0139] The above five components were mixed and placed in a water bath at 30° C for 40 minutes. Two hundred and ninety μ L were taken out of the mixture and added to 100 nmol of Seq. AB, which was previously dissolved in 80 μ L of H₂O. Then, 20 μ L of 0.8 M NaHCO₃ (pH 9.5) was added and the mixture placed in a water bath at 30° C for 1 hour. Quenching was performed by adding 39 μ L of 500 mM cysteine and letting the resultant mixture set for 30 minutes at 30° C.

[0140] Purification of the product on FPLC: column SUPERDEX® - 200, buffer 10 mM Hepes 100 mM NaCl, method 7 bank 2, Loop 1 mL. Two fractions were collected: one with the product and one with the residue. Relative absorbance PNA(Flu) ($\epsilon_{500\text{nm}} = 73000 \text{ M}^{-1}$) and GAM ($\epsilon_{278\text{nm}} = 213000 \text{ M}^{-1}$) (correction factor for PNA at 278 nm is due to the specific PNA and is calculated: 278/500 nm) was used to calculate the average conjugation ratio of PNA, GAM and DexVS70. The ratio of PNA to DexVS70 was 5.3 and the ratio of GaM to DexVS70 was 0.8.

Example 11. Exemplary embodiments of PNA1-DexVS-PNA2 conjugates

[0141]

Table 2.

Conjugate designation	ratio	PNA1	PNA1 nmol	PNA1 to DexVS	PNA2	PNA2 nmol	PNA2 to DexVS	DexVS
Conj. CA	1:9	Seq. AA	12.5	1.02	Seq. AD	100	8.2	DexVS70
Conj. CB	1:6	Seq. AC	40	1.5	Seq. AB	200	7.4	DexVS70
Conj. CC	1:16	Seq. AC	13.3	0.84	Seq. AB	200	12.7	DexVS150
Conj. CD	1:6	Seq. AC	40	2.3	Seq. AB	200	11.5	DexVS150
All conjugates were made by standard conjugation procedures of Example 17.								

Example 12. Synthesis of Anti-human-BCL2-DexVS70-PNA

[0142] Dextran (molecular weight 70 kDa) was activated with divinylsulphone to a degree of 92 reactive groups/dextran polymer, and is designated DexVS70. The antibody Anti-Human-BCL2 is designated AHB.

105 μ L DexVS70 7.5 nmol
 800 μ L AHB conc. (2.9 g/L) 15.1 nmol
 25 μ L 4 M NaCl
 32 μ L 0.8 M NaHCO₃ (pH = 9.5)

[0143] The above four compounds were mixed and placed in a water bath at 30° C for 65 minutes. From this mixture, 875 μ L was taken out and added to the indicated number of nmol of PNA in the table below; before the addition the PNA had been dissolved in the μ L of H₂O indicated in the table below. Then the number of μ LS of 0.8 M NaHCO₃ (pH

9.5) was added according to the table below. The resulting mixture was placed in a water bath at 30° C for 70 minutes. Quenching was performed by adding 6 mg of solid cysteine (0.05 M) to the mixture and letting it stand for 30 minutes at 30° C.

[0144] Purification of the product on FPLC: column SUPERDEX® - 200, buffer 10 mM Hepes 100 mM NaCl, method 7 bank 2, Loop 1 mL. Two fractions were collected: one with the product and one with the residue. Relative absorbance PNA(Flu) ($\epsilon_{500\text{nm}} = 73000 \text{ M}^{-1}$) and AHB ($\epsilon_{278\text{nm}} = 213000 \text{ M}^{-1}$) (correction factor for PNA at 278 nm is due to the specific PNA and is calculated: 278/500 nm) was used to calculate the average conjugation ratio of PNA, AHB and DexVS70.

[0145] Conjugates with different ratios PNA are shown in the following table.

Table 3.

Conjugate designation	nmol of PNA added	μL of H_2O added	μL of 0.8 M NaHCO_3 (pH 9.5) added	PNA to DexVS70	AHB to DexVS70
Conj. DA	100	75	25	9.5	1.6
Conj. DB	33	30	10	2.9	1.2
Conj. DC	67	60	20	5.6	1.1

Example 13. Solid phase synthesis and purification of Lys(Flu)-L₃₀-chr 17:14-L₃₀-Lys(Flu)-L₉₀-Lys(Flu)-L₉₀-Lys(Flu)

[0146] All Standard procedures are described in Example 18.

1. An MBHA-resin was loaded with Boc-L₃₀-Lys(Fmoc)-L₉₀-Lys(Fmoc)-L₉₀-Lys(Fmoc) using a standard loading procedure to a loading of 0.084 mmol/g.

2. To this resin, Boc-Lys(Fmoc)-L₃₀-AAC-GGG-ATA-ACT-GCA-CCT-was coupled using the peptide synthesizer machine following standard PNA solid phase chemistry. Fmoc protection groups were removed and the amino groups were labeled with fluorescein. After cleaving and precipitation the PNA was dissolved in TFA. The precipitate was washed with ether. The precipitate was dissolved in 200 μL NMP To this solution 6 mg Fmoc-Osu was added and dissolved. Next, DIPEA (9 μL) was added and the reaction was followed using MALDI-TOF mass spectrometry. After 30 minutes the reaction was finished and the PNA was precipitated and washed with ether.

[0147] HPLC after dissolving the PNA in 30 % CH_3CN and 10 % TFA/ H_2O gave three pure fractions. The fractions were pooled and lyophilized. The lyophilized PNA was then dissolved in 192 μL NMP. Piperidine (4 μL) and 4 μL DBU was added to this solution which set for 30 minutes. Analysis by MALDI-TOF mass spectrometry gave a molecular weight of 10777.

[0148] The precipitate was washed with ether and was then dissolved in 100 μL TFA. The precipitate was washed with ether and then dried using N_2 gas.

Example 14. Standard Synthesis of HRP-DexVS70-PNA Conjugate

[0149] Dextran (molecular weight 70 kDa) is activated with divinylsulfone to a degree of 92 reactive groups/dextran polymer.

192 μL DexVS70	13.7 nmol
255 μL horse radish peroxidase (HRP)	602 nmol
15 μL 4 M NaCl	
19 μL 0.8 M NaHCO_3 pH = 9.5	
119 μL H_2O	

[0150] The above five components are mixed together placed in a water bath at 30° C for 16 hours. Five hundred microliters of this mixture are added to 50 nmol PNA, which is previously dissolved in 40 μL H_2O . Then, 10 μL of 0.8 M NaHCO_3 (pH 9.5) is added. The mixture is then placed in a water bath at 30° C for 2 hours. Quenching is performed by adding 55 μL of 110 mM cysteine and letting the resultant mixture set for 30 minutes at 30° C.

[0151] Purification of the product is performed by FPLC: column SUPERDEX® - 200, buffer 10 mM Hepes 100 mM NaCl, method 7 bank 2, Loop 1 mL.

[0152] Two fractions are collected: one with the product and one with the residue. Relative absorbance HRP ($\epsilon_{404\text{nm}} = 83000 \text{ M}^{-1}$, $\epsilon_{500\text{nm}} = 9630 \text{ M}^{-1}$) and PNA(Flu) ($\epsilon_{500\text{nm}} = 73000 \text{ M}^{-1}$) is used to calculate the average conjugation ratio of HRP, PNA and DexVS70.

Example 15. Standard Synthesis of GAM-DexVS70-PNA Conjugate

[0153] Dextran (molecular weight 70 kDa) is activated with divinylsulfone to a degree of 92 reactive groups/dextran polymer (DexVS70).

105.0 μL DexVS70	7.5 nmol
57.0 μL Goat anti mouse IgG (GAM)	15 nmol
8.9 μL 4 M NaCl	
10.6 μL 0.8 M NaHCO_3 (pH = 9.5)	
144.5 μL H_2O	

[0154] The above five components are mixed and placed in a water bath at 30° C for 40 minutes. Two hundred and ninety μL is taken out of the mixture and added to 50 nmol of PNA, which is previously dissolved in 40 μL of H_2O . Then, 10 μL of 0.8 M NaHCO_3 (pH 9.5) is added and the mixture placed in a water bath at 30° C for 1 hour. Quenching is performed by adding 34 μL of 500 mM cysteine and letting the resultant mixture set for 30 minutes at 30° C.

[0155] Purification of the product on FPLC: column SUPERDEX® - 200, buffer 10 mM Hepes 100 mM NaCl, method 7 bank 2, Loop 1 mL. Two fractions are collected: one with the product and one with the residue. Relative absorbance PNA(Flu) ($\epsilon_{500\text{nm}} = 73000 \text{ M}^{-1}$) and GAM ($\epsilon_{278\text{nm}} = 213000 \text{ M}^{-1}$) (correction factor for PNA at 278 nm is due to the specific PNA and is calculated: 278/500 nm) was used to calculate the average conjugation ratio of PNA, GAM and DexVS70.

Example 16. Standard synthesis of PNA1-DexVS70-PNA2

[0156] Dextran (molecular weight 70 kDa) is activated with divinylsulfone to a degree of 92 reactive groups/dextran polymer. PNA1 (100 nmol) is dissolved in 140 μL of DexVS70 (10 nmol). To this mixture 12.5 μL of PNA2 (12.5 nmol) dissolved in H_2O is added, and then 30 μL of NaHCO_3 (pH 9.5) is added and the solution mixed. The resultant mixture is placed in a water bath at 30° C for 35 minutes. Quenching was performed by adding 18.3 μL of 500 mM cysteine in Hepes and letting this mixture set for 30 minutes at 30° C.

[0157] Purification of the product on FPLC: column SUPERDEX® - 200, buffer 10 mM Hepes 100 mM NaCl, method 7 bank 2, Loop 1 mL. Two fractions are collected: one with the product and one with the residue. Relative absorbance PNA(Flu) ($\epsilon_{500\text{nm}} = 73000 \text{ M}^{-1}$) and the proportion between the two PNA's is used to calculate the average conjugation ratio of PNA, PNA and DexVS70.

Example 17. Synthesis of the Boc-PNA-I(O-Bz)-monomer

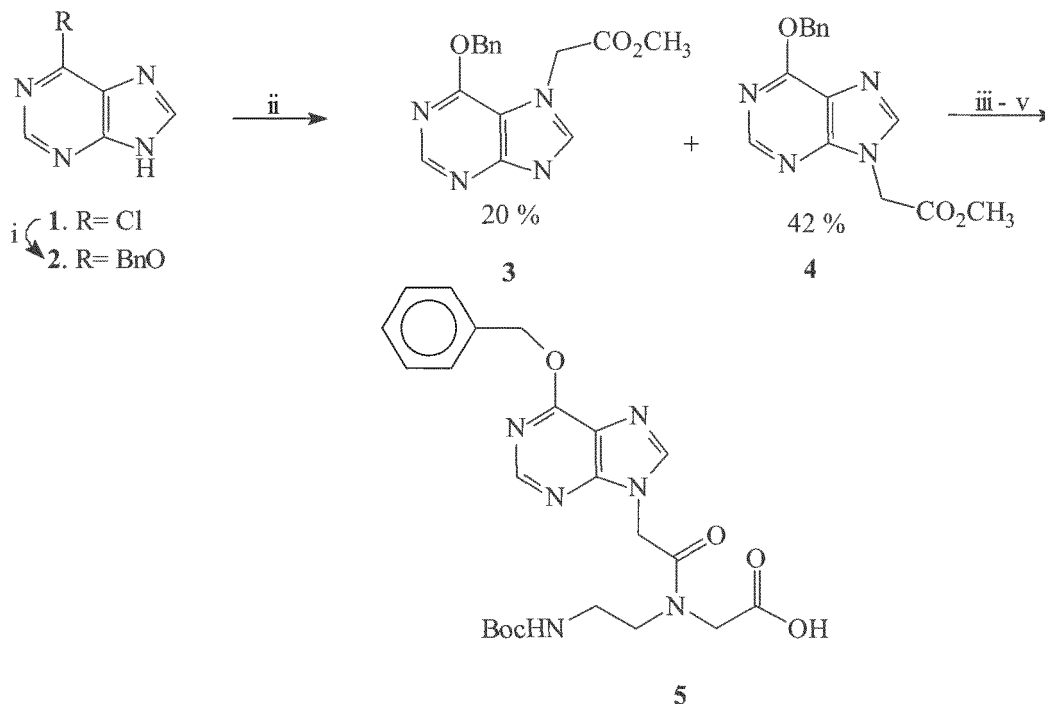
[0158] 6-Benzyloxypurine. Sodiumhydride (60 % Dispersion in mineral oil; 3,23g; 80 mmol) was slowly added to benzyl alcohol (30 ml; 34,7 mmol). After the addition of more benzyl alcohol (10 ml) and 6-chloropurine (5,36 g;). The reaction mixture was heated to 100°C for 4 hours. When the reaction mixture has reached room temperature, water (1ml) was slowly added. 6-Benzyloxypurine was precipitated by the addition of acetic acid (4,6 ml) and diethylether (550 ml). The precipitate was separated by filtration (11,72 g). Re-crystallization from ether gave (4,78 g; 65,4 %). Melting point was 175 - 177 °C (litt. 170-172 °C)[Ramazaeva N., 1989 #473] 1 H-NMR (DMSO-d6): 8,53 (1 H,s); 8,39 (1 H, s); 7,54-7,35 (5H, m); 5,62 (2H,s).

[0159] Methyl (6-(Benzyloxy)purin-9-yl) acetate. 6-Benzyloxypurine (4,18 g; 18,5 mmol) was added to a suspension of potassium carbonate (3,1 g; 22,4 mmol) in DMF (100 ml). After 15 min., bromoacetic acid methyl ester (1,93 ml; 20,4 mmol) was added. The reaction was monitored by TLC in butanol:acetic acid:water 4:1:1. Upon completion, the reaction mixture was partitioned between water (600 ml) and ethyl acetate (600 ml). The organic phase was dried over magnesium sulfate and evaporated to a volume of ~10 ml and precipitated with pet. ether. The two products were separated by column chromatography using ethyl acetate as the solvent. The products were precipitated in pet. ether. Yield: 2,36 g (43 %). Melting point: 111,5-115°C. UV $\lambda_{\text{max}} = 250 \text{ nm}$ (9-alkylated); $\lambda_{\text{max}} = 260 \text{ nm}$ (7-alkylated). 1 H-NMR (DMSO-d6): 8,60 (1 H, s); 8,43 (1 H,s); 7,6-7,35 (5H, m); 5,69 (2H, s); 5,26 (2H, s); 3,75 (3H, s).

[0160] (6-(Benzyloxy)purin-9-yl) acetic acid. Methyl (6-(Benzyloxy)purin-9-yl) acetate (2,10 g; 7,0 mmol) was dissolved in methanol (70 ml) and 0,1 M NaOH (85 ml) is added. After 15 min. the pH of the reaction mixture was lowered by addition of 0,1 M HCl (~ 80 ml) to pH 3. The precipitate was separated from the mixture by filtration and washed with water and ether. Yield: 1,80 g (90,2 %). 1 H-NMR (DMSO-d6): 8,55 (1 H,s); 8,37 (1 H,s); 7,55-7,30 (5H, m); 5,64 (2H,

s); 5,09 (2H, s).

[0161] N-((6-(Benzyloxy)purin-9-yl) acetyl)-N-(2-Boc-aminoethyl)glycine. Ethyl N-(2-Boc-aminoethyl)glycinate (0,285 g; 1,15 mmol), (6-(benzyloxy)purin-9-yl) acetic acid (0,284 g; 1,0 mmol) and 3-hydroxy-1,2,3 benzotriazin-4(3H)-one (0,180 g; 1,1 mmol) was dissolved in dichloromethane/dimethylformamide 1:1 (10 ml). After addition of dicyclohexylcarbodiimide (0,248 g; 1,2 mmol) the reaction was left over night. The precipitate was removed by filtration. The organic phase was extracted twice with saturated sodium bicarbonate, dried with magnesium sulfate and evaporated to a oil. Column purification on silica using dichloromethane with 0-5 % methanol as elutant yields the monomer ester which was dissolved in methanol (10 ml). Then, 0,1 M NaOH (12 ml) was added. After 30 min the reaction was filtered and pH adjusted with saturated KHSO₄ / water (1:3) to 2,7. The water phase was extracted twice with ethyl acetate (2 x 100 ml). The combined organic phases were dried over magnesium sulfate and evaporated to a volume of 10 ml. Precipitation with pet. ether yielded the monomer (0,15 g; 31 %). ¹H-NMR (DMSO-d₆): 8,51 (1H, s); 8,23 (1H, s); 7,6-7,3 (5H, m); 5,64 (2H, s); 5,31 (ma.) + 5,13 (mi.) (2H, s); 4,23 (mi.) + 3,98 (ma.) (2H, s); 3,55-3,00 (4H, m); 1,36 (9H, s).



The synthesis of the hypoxanthine PNA monomer. (i) BnOH, NaH (ii) K₂CO₃, BrCH₂CO₂CH₃ (iii) OH- (iv) DCC, Dhbt-OH, Boc-aeg-OEt (v) OH-

[0162] The Boc-PNA-Diaminopurine-(N6-Z)-monomer was prepared according to Gerald Haaima, Henrik F. Hansen, Leif Christensen, Otto Dahl and Peter E. Nielsen; Nucleic Acids Research, 1997, Vol 25, Issue 22 4639-4643.

[0163] The Boc-PNA-2-Thiouracil-(S-4-MeOBz)-monomer was prepared according to Jesper Lohse, Otto Dahl and Peter E. Nielsen; Proceedings of the National Academy of Science of the United States of America, 1999, Vol 96, Issue 21, 11804-11808.

[0164] The Boc-PNA-Adenine-(Z)-monomer was from PE Biosystems catalog GEN063011.

[0165] The Boc-PNA-Cytosine-(Z)-monomer was from PE Biosystems cat. GEN063013.

[0166] The Boc-PNA-Guanine-(Z)-monomer was from PE Biosystems cat. GEN063012.

[0167] The Boc-PNA-Thymine-monomer was from PE Biosystems cat. GEN063010.

[0168] IsoAdenine (2-aminopurine) may be prepared as a PNA-monomer by 9-N alkylation with methylbromoacetate, protection of the amino group with benzylchloroformate, hydrolysis of the methyl ester, carbodiimide mediate coupling to methyl-(2-Boc-aminoethyl)-glycinate, and finally hydrolysis of the methyl ester.

[0169] 4-thiouracil may be prepared as a PNA-monomer by S-protection with 4-methoxy-benzylchloride, 1-N alkylation with methylbromoacetate, hydrolysis of the methyl ester, carbodiimide mediate coupling to methyl-(2-Boc-aminoethyl)-glycinate, and finally hydrolysis of the methyl ester.

[0170] Thiocytosine may be prepared as a PNA monomer by treating the Boc-PNA-cytosine(Z)-monomer methyl ester with Lawessons reagent, followed by hydrolysis of the methyl ester.

[0171] A number of halogenated bases are commercially available, and may be converted to PNA monomers analogously to the non-halogenated bases. These include the guanine analog 8-bromo-guanine, the adenine analogs 8-

bromo-adenine and 2-fluoro-adenine, the isoadenine analog 2-amino-6-chloro-purine, the 4-thiouracil analog 5-fluoro-4-thio-uracil, and the 2-thiouracil analog 5-chloro-2-thiouracil.

[0172] Boc-PNA-Uracil monomers were first described in "Uracil og 5-bromouracil I PNA," a bachelor project by Kristine Kilså Jensen, Københavns Universitet 1992.

Example 18. Miscellaneous Standard Procedures

[0173]

a. Loading of resins. P-methyl-BHA-resin (3 g) is loaded with Boc-Lys(Fmoc)-OH 15 mmol/g resin. The lysine is dissolved in NMP and activated with 0.95 equivalents (eq.) HATU and 2 eq. DIPEA. After loading the resin, it is capped by adding a solution of (Ac)₂O / NMP/ pyridine (at a ratio of 1/2/2) and letting it set for at least 1 hour or until Kaiser test was negative. After washing with DCM, the resin is dried in a dessicator. Quantitative Kaiser test typically gives a loading of 0.084 mmol/g.

b. Amino Acid Couplings. The Boc protection group is removed from the resin with TFA / m-cresol (at a ratio of 95/5) 2x5 min. The resin is then washed with DCM, pyridine and DMF before coupling with the amino acid, which is dissolved in NMP in a concentration between 0.2 and 0.4 M and activated with 0.95 eq. of HATU and 2 eq of DIPEA for 2 minutes. The coupling is complete when the Kaiser test is negative. Capping occurring by exposing the resin for 3 minutes to (Ac)₂O / pyridine / NMP (at a ratio of 1/2/2). The resin is then washed with DMF and DCM

c. Boc-L₃₀₀-Lys(Fmoc)-resin. To the loaded Boc-Lys(Fmoc)-resin, L₃₀-Linker in a concentration of 0.26 M was coupled using standard amino acid coupling procedure. This was done 10 times giving Boc-L₃₀₀-Lys(Fmoc)-resin.

d. PNA solid phase. On a peptide synthesizer (ABI 433A, Applied Biosystems) PNA monomers are coupled to the resin using standard procedures for amino acid coupling and standard PNA chemistry. Then the resin is handled in a glass vial to remove protections groups and to label with either other amino acids or flourophores.

[0174] Removal of the indicated protection groups is achieved with the following conditions:

Boc: TFA / m-cresol (at a ratio of 95/5) 2x5 min.

Fmoc: 20 % piperidine in DMF 2x5 min.

Dde: 3 % hydrazine in DMF 2x5 min.

[0175] When the synthesis is finished, the PNA is cleaved from the resin with TFA/TFMSA/m-cresol/thioanisol (at a ratio of 6/2/1/1). The PNA is then precipitated with ether and purified on HPLC. MALDI-TOF mass spectrometry is used to determine the molecular weight of the product.

e. Labeling with fluorescein. 5(6)-carboxy fluorescein is dissolved in NMP to a concentration of 0.2 M. Activation is performed with 0.9 eq. HATU and 1 eq. DIPEA for 2 min before coupling for at least 2x20 min or until the Kaiser test is negative.

Example 19. PNA With Positive and Negative Loadings

[0176] In order to make better conjugations at one time we tried to give the PNA a loading. Both PNA's were made by PNA standard procedures (See Example 18).

1. Flu-L₃₀-Glu-TCA-AGG-TAC-A-Glu-L₃₀₀-Lys(Cys)

Glu = glutamate has negative loadings and for the easiness the PNA is designated -A4-

2. Flu-L₃₀-Lys(Me)₂-TGT-ACC-TTG-A-Lys(Me)₂-L₃₃₀-Lys(cys)

Lys(Me)₂ = Boc-Lys(Me)₂-OH has positive loadings and the PNA is designated +T+

Table 4.

name	number	HRP	GaM	equiv.	HRP/Dex	GaM/Dex	PNA/Dex

(continued)

name	number	HRP	GaM	equiv.	HRP/Dex	GaM/Dex	PNA/Dex
-A4-	D 13041	D 13050		9	12.3		0.13
-A4-	D 13041		D 13060	7		0.94	0.66
+T4+	D 13042	D 13058		9	13.5		0.19
+T4+	D 13042		D 13056	7		1.42	0.45
As it is shown in the scheme, PNAs with loading are not good at coupling.							

Example 20. Target Detection: Procedures used in the Examples Below1. Fixation of biological samples

[0177] Tonsil tissue samples were fixed in neutral buffered formalin, NBF (10 mM NaH₂PO₄/ Na₂HPO₄, pH 7.0), 145 mM NaCl, and 4% formaldehyde (all obtained from Merck, Whitehouse Station, NJ). The samples were incubated overnight in a ventilated laboratory hood at room temperature.

2. Sample dehydration and paraffin embedding

[0178] The tissue samples were placed in a marked plastic histocapsule (Sakura, Japan). Dehydration was performed by sequential incubation in 70% ethanol twice for 45 min, 96% ethanol twice for 45 min, 99% ethanol twice for 45 min, and xylene twice for 45 min. The samples were subsequently transferred to melted paraffin (melting point 56-58°C) (Merck, Whitehouse Station, NJ) and incubated overnight (12-16 hours) at 60°C. The paraffin-infiltrated samples were transferred to fresh warm paraffin and incubated for an additional 60 min prior to paraffin embedding in a cast (Sekura, Japan). The samples were cooled to form the final paraffin blocks. The marked paraffin blocks containing the embedded tissue samples were stored at room temperature in the dark.

3. Cutting, mounting and deparaffination of embedded samples

[0179] The paraffin blocks were cut and optionally also mounted in a microtome (0355 model RM2065, Feather S35 knives, set at 5.0 micrometer; Leica, Bannockburn, IL). The first few millimeters were cut and discarded. Paraffin sections 4-6 micrometers thick were then cut and collected at room temperature. The sections were gently stretched on a 45-60°C hot water bath before being mounted onto marked microscope glass slides (SUPERFROST® Plus; Fisher, Medford, MA), two tissue sections per slide. The slides were then dried and baked in an oven at 60°C. The slides were deparaffinated by incubating twice in xylene for 5 min ± 2 min twice, then in 96% ethanol for 2min +/- 30 sec, then twice in 70% ethanol for 2 min +/- 30 sec, and then once in Tris-buffered saline with TWEEN® (called herein TBST) for 5 min. TBST comprises 50 mM Tris adjusted to pH 7.6 with HCl; 150 mM NaCl; 0.05 % TWEEN®20. The slides were deparaffinated by subsequently incubation in xylene twice for 5 min ± 2 min, 96% ethanol twice for 2min +/- 30 sec and 70% ethanol twice for 2 min +/- 30 sec. The slides were immersed in deionized water and left for 1 to 5 min.

4. Endogenous peroxidase blocking

[0180] Samples were incubated with a 3% hydrogen peroxide solution for 5 min. to quench endogenous peroxidase activity, followed by washing in deionized water for 1 to 5 min.

5. Antigen retrieval by microwave oven

[0181] Antigens in the sample were retrieved by immersing the slides in a container containing Antigen Retrieval Solution, pH 6.0 (DakoCytomation code No. K5204 Vial 7 or optional code No. K5205 Vial 7). The container was closed with a perforated lid and placed in the middle of a microwave oven and left boiling for 10 min. The container was removed from the oven and allowed to cool at room temperature for 20 min. The samples were rinsed in deionized water.

6. Antigen retrieval by water bath incubation

[0182] Antigens in the sample were retrieved by immersing the slides in a beaker containing Antigen Retrieval Solution,

pH 6.0 (DakoCytomation code No. K5204 Vial 7 or optional code No. K5205 Vial 7). The samples were incubated for 40 min in a water bath at 95-100°C. The beaker was removed from the water bath and allowed to cool at room temperature for 20 min. The samples were rinsed in deionized water.

7. Water-repellent barrier to liquids by DakoCytomation Pen

[0183] To ensure good coverage of reagent on the tissue sample, the area on the slide with tissue was encircled with a silicone rubber barrier using DakoCytomation Pen (DakoCytomation code No. 2002). The slides were transferred to a rack and placed in a beaker containing Tris-buffered saline with TWEEN® (called herein TBST) and left for 5 min. TBST comprises 50 mM Tris adjusted to pH 7.6 with HCl; 150 mM NaCl; 0.05 % TWEEN®20.

8. Application of a primary antibody

[0184] Monoclonal Mouse anti-Human Cytokeratin (DakoCytomation code No. M3515) diluted 1:900 in ChemMate™ Antibody Diluent (DakoCytomation code No. S2022) was applied on the tissue samples and incubated for 30 min in a humid chamber at ambient temperature. The slides were individually rinsed and then washed in TBST for 5 min.

9. Application of three primary antibodies

[0185] Monoclonal Mouse Anti-Human Cytokeratin (DakoCytomation code No. M3515) diluted 1:300, 1:900 and 1:1600; monoclonal Mouse Anti-Human CD20cy (DakoCytomation code No. M0755) diluted 1:2000, 1:8000 and 1:14000; and monoclonal Mouse Anti-Human Ki-67 Antigen (DakoCytomation code No. M7240) diluted 1:400, 1:1200 and 1:2400 were used. The antibodies were diluted in ChemMate™ Antibody Diluent (DakoCytomation code No. S2022), applied on the tissue samples, and incubated for 30 min in a humid chamber at ambient temperature. The slides were individually rinsed and washed in TBST for 5 min.

10. Application of an Antibody / Dextran / PNA1 Conjugate Recognition Unit

[0186] Antibody / Dextran / PNA1 conjugate recognition unit is also called "PNA1 conjugate" in the examples that follow. The PNA1 conjugate comprises 70,000 molecular weight dextran. Table 1 summarizes PNA1 conjugates based on a secondary antibody: goat anti-mouse Ig, called herein GAM (DakoCytomation code No. Z0420). Table 2 summarizes PNA1 conjugates based on a primary antibody: mouse anti-human BCL2 oncoprotein, such as Clone 124 (DakoCytomation code No. M0887). The primary antibody was protein A-purified prior to conjugation. The conjugates were diluted in BBA (50 mM Tris adjusted to pH 7.6 with HCl; 150 mM NaCl; 2% BSA; 0.02% bronidox; 2.44 mM 4-aminoantipyrin) and were applied on the tissue sample in a range of dilutions, then incubated for 30 min in a humid chamber at ambient temperature. The slides were individually rinsed and washed in TBST for 5 min.

Table 5. PNA1 conjugates useful in indirect recognition of targets: GAM / Dextran / PNA1

Conjugate No.	Sequence	μM Dex	GAM / Dex	PNA1 / Dex
D14120	AGA CPT TPG DPT	1.25	1.1	4.3
D14102	GTP TAA TTP PAG	1.02	1.0	9.1
D14096	GTP TAD TTP PAG	1.15	1.4	4.2
D14083	U _s GU _s DPP TTG D	0.87	0.8	5.3
D13171	U _s GU _s DPP TTG D	1.21	1.0	7.5
D13161	TTG APP TTA G	2.11	1.1	6.0
D13150	TGT APP TTGA	2.20	1.1	4.2
D13102	TGT ACC TTGA	2.53	1.1	2.5
D12102	TGT ACC TTGA	2.50	1.3	4.5

Table 6. PNA1 conjugates for direct recognition of targets: anti-BCL2 / Dextran / PNA1

Conjugate No.	Sequence	μM Dex	Ab / Dex	PNA1 / Dex
D14128	$\text{U}_5\text{GU}_5\text{ DPP TTG D}$	0.8	1.1	5.6
D14126	$\text{U}_5\text{GU}_5\text{ DPP TTG D}$	1.0	1.2	2.9
D14122	$\text{U}_5\text{GU}_5\text{ DPP TTG D}$	1.1	1.6	9.5

In the above tables, the letters A, C, G, U, and T, stand for the natural bases adenine, cytosine, guanine, uracil, and thymine. P stands for pyrimidinone, D for 2,6-diaminopurine, and U_5 for 2-thiouracil.

11. Fixation of PNA1-conjugate with 1% glutardialdehyde

[0187] The samples were washed in deionized water for 30 sec. Then, 1 % glutardialdehyde (Merck Art. No. 820603), called herein GA, diluted in 22 mM calcium phosphate buffer, pH 7.2, was applied, and the samples were incubated for 10 min in a humid chamber at ambient temperature. The samples were washed in deionized water for 30 sec and in TBST for 5 min.

12. Application of a PNA¹-PNA²/ Dextran Conjugate Adaptor Unit

[0188] PNA¹-PNA²/ Dextran conjugate is also called "PNA¹-PNA²" in the following examples. Table 7 summarizes the compositions of PNA¹-PNA² conjugates. PNA¹ is complementary to the PNA1 conjugate, and PNA² is complementary to the PNA2 conjugates D14079 and D13155 described in step 13 below. The sequence of PNA¹ is $\text{CU}_5\text{G}_5\text{G}_5\text{DD TU}_5\text{D G}_5\text{DC}$ and the sequence of PNA² is $\text{U}_5\text{GU}_5\text{ DPP TTG D}$, in which U_5 stands for 2-thio-uracil, G_5 stands for 2-amino-6-thioxopurine, D stands for diaminopurine, and P stands for pyrimidinone. The conjugates, diluted in BBA, were applied to the tissue samples in a range of dilutions, and the samples were then incubated for 30 min in a humid chamber at ambient temperature. The samples were individually rinsed and washed in TBST for 5 min. When testing a PNA¹-PNA²conjugate, fixed concentrations of 0.08 μM PNA1 and 0.05 μM PNA2 were used.

Table 7. PNA¹-PNA²/ Dextran conjugates

Conjugate No.	Molecular weight of dextran	PNA ¹ / dex	PNA ² / dex	μM PNA ¹
D14119	150.000	2.3	11.5	4.2
D14106	150.000	0.8	12.7	1.3
D14104	70.000	1.5	7.5	3.9

13. Application of Horse Radish Peroxidase / Dextran / PNA2 Conjugate Detection Unit

[0189] Horse Radish Peroxidase (HRP) / Dextran / PNA2 conjugates are also called "PNA2 conjugate" in the examples that follow, and are listed in table 8. The PNA2 conjugates comprise 70.000 Da molecular weight dextran. The conjugates diluted in BBA were applied to the tissue samples in a range of dilutions, and samples were incubated for 30 min in a humid chamber at ambient temperature. The samples were individually rinsed and washed twice in TBST for 5 min.

Table 8. PNA2 conjugates: HRP / Dextran / PNA2

Conjugate No.	Sequence	μM PNA	HRP / Dex	PNA2 / Dex
D14133	TCD DII TAC A	1.6	14.0	1.0
D14111	$\text{DG}_5\text{T CG}_5\text{D DG}_5\text{G U}_5\text{CU}_5$	3.9	11.4	2.1
D14110	$\text{DGT CG}_5\text{D DG}_5\text{G U}_5\text{CU}_5$	3.0	12.6	1.6
D11089	$\text{CU}_5\text{G}_5\text{ G}_5\text{DD TU}_5\text{D G}_5\text{DC}$	2.1	14.1	1.5
D14086	$\text{U}_5\text{CG}_5\text{ G}_5\text{DD TU}_5\text{D GDC}$	1.9	11.0	1.0
D14079	TCD DG_5G_5 TAC A	1.9	12.2	1.2
D13159	CTA AG_5G_5 TCA A	1.9	12.9	1.3

(continued)

Conjugate No.	Sequence	μM PNA	HRP / Dex	PNA2 / Dex
D13155	TCD DG _s G _s TAC A	2.4	12.7	1.6
D13148	TCA AG _s G _s TAC A	1.9	11.6	0.8
D13122	CTA AGG TCA A	3.2	13.0	2.1
D13108	GTG TGT GT	4.3	12.0	2.3
D13106	TCA AGG TAC A	2.6	12.4	1.3
D12120	TCD DGG TAC A	1.0	18.3	0.6
D12094	TCA AGG TAC A	3.0	14.6	0.9
In Table 8, in addition to the nucleobase letter schemes provided for Tables 5-7, I stands for inosine.				

14. Application of diaminobenzidine chromogenic substrate solution

[0190] The diaminobenzidine chromogenic substrate solution, DAB+ (DakoCytomation code No. K3468) was applied on the tissue samples, and the samples were incubated for 10 min in a humid chamber at ambient temperature. The samples were washed with deionized water for 5 min.

15. Counterstaining with Hematoxylin

[0191] The tissue samples were immersed in Mayers Hematoxylin (Bie & Berntsen Code No. LAB00254) for 3 min, rinsed in tap water for 5 min, and finally rinsed with deionized water.

16. Cover slipping

[0192] Cover slips were applied to the tissue samples using the aqueous mounting media, Faramount (DakoCytomation code No. S3025).

17. Evaluation of the performance

[0193] The tissue staining was examined in a bright field microscope at 10x, 20x or 40x magnification. Both the specific and the non-specific staining intensity were described with a score-system using the range 0 to 3+ with 0.5+ score interval. ChemMate™ EnVision™ Detection kit Rabbit/Mouse (DakoCytomation code No. K5007 bottle A) was used as a reference, and was included in all experiments for testing in parallel with the PNA conjugates. K5007 was used according to manufacturer's instructions. The antibodies were used in the following dilutions: M3515 at 1:900, M0755 at 1:8000, and M7240 at 1:1200. The staining intensity of the K5007 reference using the primary antibody M3515 diluted 1:900 was set to 2+ in order to compare and assess the staining result of the PNA conjugate tested. If the reference deviated more than ± 0.5 , the test was repeated.

[0194] In the examples, the various visualization system combinations of the invention were tested on routine tissue samples. The staining performance was compared with a reference visualization system, using EnVision™ and a very dilute antibody from DakoCytomation. The practical dynamic range of quantitative IHC may be narrow, and e.g. strongly stained (+3) tissues are not easy to compare with respect to intensity. Therefore, on purpose, the staining intensity of the reference system was adjusted to be approximately +2. This was done in order to better monitor and compare differences in staining intensity with the system of the invention.

Example 21: Protocol for fast evaluation of non-specific binding of PNA2 conjugates

[0195] The protocol allowed for a quick test of PNA2 conjugates for non-specific staining. Tonsil tissues were taken through the steps 1-5, 7, 13-14, 15 (in which the slides were immersed in a bath of Hematoxylin Mayer for 1 min.), and 16-17, above. The conjugates to be tested were diluted to the final concentrations 0.05 μM and 0.2 μM . As references, two PNA2 conjugates were used in the final concentration 0.05 μM . The first reference, for example, PNA2, D13108, was known to give non-specific nuclear staining, and so was used as a positive control. The second reference, for example, PNA2, D13155, was known not to give any non-specific nuclear staining, and was used as a negative control. In general, 250 μL of each reagent was applied unless otherwise specified.

Protocol for test of a PNA pair with one antibody

[0196] Tonsil tissues were taken through the steps 1-4, 6-8, 10, 11, and 13-17 above. Step 11 was left out for the tonsils not fixed with 1% GA. In general, 250 μ L of each reagent was applied unless otherwise specified.

Protocol for test of 3-laver PNA conjugates

[0197] Tonsil tissues were taken through the steps 1-4, 6-8, and 10-17 above. Step 11 was left out for the tonsils not fixed with 1% GA. In general, 250 μ L of each reagent was applied unless otherwise specified.

Protocol for test of a PNA pair with 3 antibodies

[0198] Tonsil tissues were taken through the steps 1-4, 6, 7, 9 -11, and 13-17 above. Step 11 was left out for the tonsils not fixed with 1 % GA. A further negative control, mouse IgG1 (DakoCytomation code No. X0931) diluted 1:300 in S2022 was included the protocol for the PNA conjugates. In general, 250 μ L of each reagent was applied unless otherwise specified.

Example 22. Testing and Selection of PNA pairs

[0199] Conjugates comprising example PNA segments were tested for their ability to specifically hybridize according to the invention. Tonsil tissues were taken through the steps 1-4, 6-8, 10 and 13-17 above. K5007 was included as a reference to secure the level of the staining. The concentration of the conjugates was 0.08 μ M for PNA1 and 0.05 μ M for PNA2.

[0200] The results listed in Table 10 show the staining intensities for a representative number of PNA pairs tested. The PNA pairs did not demonstrate any non-specific binding. The specific staining, in general, was directly proportional to the number of hydrogen bonds involved in the base-pairing. It was important that each PNA did not interact with itself. Substitution of T (thymine) with U_s (2-thiouracil) in some PNAs could prevent such intra-PNA interactions. The unspecific staining intensity was increased by substituting an A with a D. On the other hand, we also observed that replacement of one G (guanine) with G_s (2-amino-6-thioxopurine) in the same PNA could circumvent the unspecific binding introduced by D. For instance, the staining of the PNA1 conjugate D14102 was improved by substituting the D in D14096 with an A in D14102. As is apparent from Table 9, this small change resulted in an increase of the specific staining score by 1+.

Table 9.

PNA1	PNA2	Specific staining intensity	Non-specific staining intensity
D13161	D13159	2	0
D14083	D14079	2.5	0
D14096	D14089	1.5	0
D14102	D14089	2.5	0
D14120	D14114	1.5	0

Example 23. The effect of base substitution on PNA-specific binding intensities

[0201] The PNA pair D13102-D13106 was used as a starting point for further investigation of introducing base substitutions in either PNA1 or PNA2 conjugates. Tonsil tissues were taken through the steps 1-4, 6-8, 10 and 13-17. Each of the three different PNA1 conjugates was tested with each of the three different PNA2 conjugates. The concentration of the conjugates used was 0.08 μ M for PNA1 and 0.05 μ M for PNA2.

Table 10.

PNA2: PNA1:	D13106 TCAAGGTACA	D13148 TCAAG _s G _s TAC A	D13155 TCD DG _s G _s TAC A
D13102 TGT ACC TTG A	2.5	0	2
D13150 TGTAPPTTG A	2.5	0.5	3
D13171 U _s GU _s DPP TTG D	3	2.5	3

[0202] Table 10 shows the effect of base substitutions on the specific binding between paired PNA variants. No non-specific binding was observed. D13102 tested with D13106 gave a specific staining of 2.5+. Replacement of 2 G's with 2 G_s's (D13148) resulted in the abolishment of specific staining, but by introducing 2 D's instead of 2 A's (D13155) achieved a specific staining of 2+. When the 2 C's in D13102 were replaced with 2 P's (D13150) and tested with D13106, the specific staining was unchanged at 2.5+, despite the lower number of hydrogen bonds as compared to the PNA-pair D13102-D13106. Test of D13150 with D13148 resulted in a reduced specific staining of 0.5+, whereas specific staining to 3+ was observed for the D13150-D13155 pair. The replacement in D13150 of 2 A's with 2 D's and of 2 T's with 2 U_s's (D13171) resulted in improved specific binding compared to D13106. This modified PNA1 was now able to bind specifically to D13148 with a score of 2.5+, and also bound to D13155.

[0203] This experiment clearly demonstrates the use of PNA pairs in the present invention. Furthermore, it shows the ability of fine tuning the specific binding by introducing base substitutions using either natural as well as non-natural bases.

Example 24. Test of cross reactivity

[0204] The two PNA-pairs, D13150-D13155 and D13161-D13159, were tested for cross-reactivity. Tonsil tissues were taken through the steps 1-4, 6-8, 10, and 13-17. The concentration of conjugates used was 0.16 μ M for PNA1 and 0.1 μ M for PNA2.

[0205] As apparent from Table 11, PNA1 D13150 did not cross react with PNA2 D13159, but PNA1 D13161 cross reacted with PNA2 D13155. We therefore excluded the PNA pair D13161-D13159 due to the cross-reaction between D13161 and D13155. No non-specific staining was observed.

Table 11: Test of specific binding and cross reactivity

PNA2:	D13155	D13159
PNA1:		
D13150	2.0	0
D13161	1	1.5

Example 25. Test of cross reactivity

[0206] Three PNA-pairs, D14083-D14079, D14102-D14089 and D14120-D14114 were tested for cross-reactivity. Tonsil tissues were taken through the steps 1-4, 6-8, 10, and 13-17. The concentration of the conjugates used was 0.08 μ M for PNA1 and 0.05 μ M for PNA2.

[0207] The PNA conjugates listed in Table 12 only bound to their complementary partner and did not cross react to any of the other PNA conjugates tested. No non-specific staining was observed. See Table 12 below.

Table 12: Test of specific binding and cross reactivity

PNA2:	D14079	D14089	D14114
PNA1:			
D14083	1.5	0	0
D14102	0	1	0
D14120	0	0	1.5

Example 26.

[0208] Two PNA pairs, D14083-D14079 and D14096-D14089, were tested at different PNA2 concentrations for the purpose of determining the optimal concentration of PNA2 conjugates. The concentrations used were 0.08 μ M for PNA1 conjugates and 0.025; 0.05; 0.1 and 0.2 μ M for PNA2 conjugates.

[0209] Tonsil tissues were taken through the steps 1-4, 6-8, 10, and 13-17. The optimal concentration of the PNA2 conjugate was 100 nM. See Table 13 below.

Table 13: Determination of PNA2 conjugate concentration.

PNA1	1% GA fixation of PNA1	PNA2	Specific staining			
			0.025 μ M	0.05 μ M	0.1 μ M	0.2 μ M
D14083	-	D14079	1.5	2	3	2.5
D14096	-	D14089	0.5	1	2	1.5

[0210] Tonsil tissues were taken through the steps 1-4, 6-8, 10, 11, and 13-17. Step 11 was omitted for tissues not fixed with 1% GA.

[0211] Fixation of PNA1 conjugates with 1% GA resulted in a stronger specific staining than without fixation and the optimal concentration of the PNA2 conjugate was now determined to be 50 nM. See Table 14 below.

Table 14: Effect of 1% GA fixation on the determination of PNA2 conjugate concentration.

PNA1	1% GA fixation of PNA1	PNA2	Specific staining			
			0.025 μ M	0.05 μ M	0.1 μ M	0.2 μ M
D14083	-	D14079	2	2	2.5	2.5
D14083	+	D14079	2.5	3	2.5	3

Example 27. Standard synthesis of an alkaline phosphatase - dextran - PNA conjugate molecule

[0212] Alkaline Phosphatase ("AP") (from Calf Intestine, EIA grade) was dialyzed overnight against 2mM HEPES, pH 7.2; 0.1 M NaCl; 0.02mM ZnCl₂. Dextran (molecular weight 70kDa) was activated with divinylsulfone to a degree of 92 reactive groups per dextran polymer (DexVS70).

[0213] The three components below were mixed together and placed in a water bath at 40°C for 30 minutes.

192.0 μ L DexVS70 13.7 nmol
 41.0 μ L PNA 41 nmol PNA dissolved in H₂O
 6.0 μ L 1 M NaHCO₃

[0214] 108.0 μ L of the DexVS70-PNA conjugate was taken out and added to a mixture of:

160.0 μ L AP 43.4 nmol
 7.7 μ L 1M NaHCO₃
 30.6 μ L 20mM Hepes, pH 7.2; 1 M NaCl; 50mM MgCl₂; 1 mM ZnCl₂

[0215] The mixture was placed in a water bath at 40°C for 3 hours. Quenching was performed by adding 30.6 μ L of 0.1 M ethanolamine and letting the mixture stand for 30 minutes in water bath at 40°C. The product was purified on FPLC with:

Column Superdex-200, buffer: 2mM HEPES, pH 7.2; 0.1 M NaCl; 5mM MgCl₂; 0.1 mM ZnCl₂. Two fractions were collected, one with the product and one with the residue.

[0216] In comparison to the experiment described above, another conjugate was made with extended conjugation time. The three components below were mixed together and placed in a water bath at 40°C for 30 minutes.

192.0 μ L DexVS70 13.7 nmol
 41.0 μ L PNA 41nmol PNA dissolved in H₂O
 6.0 μ L 1M NaHCO₃

[0217] 108.0 μ L of the DexVS70-PNA conjugate was taken out and added to a mixture of:

160.0 μ L AP 43.4 nmol

(continued)

7.7 μ L 1M NaHCO₃
 30.6 μ L 20mM Hepes, pH 7.2; 1M NaCl; 50mM MgCl₂; 1 mM ZnCl₂

[0218] The mixture was placed in a water bath at 40°C for 5 hours. Quenching was performed by adding 30.6 μ L 0.1 M Ethanolamine and letting the mixture stand for 30 minutes in water bath at 40°C. Purification of the product on FPLC: Column Superdex-200, buffer: 2mM Hepes, pH 7.2; 0.1 M NaCl; 5mM MgCl₂; 0.1 mM ZnCl₂. Two fractions were collected: One with the product and one with the residue.

[0219] Relative absorbance PNA(Flu) ($\epsilon_{500\text{nm}} = 73000\text{M}^{-1}$) and AP ($\epsilon_{278\text{nm}} = 140000\text{M}^{-1}$. Corrected for absorbance from PNA at 278nm, this correction factor is due to the specific PNA and it is calculated: 278/500nm) was used to calculate the average conjugation ratio of PNA, AP and DexVS70.

AP-DexVS70-PNA, 3hrs:

PNA/DexVS70: 1.8
 AP/DexVS70: 1.8

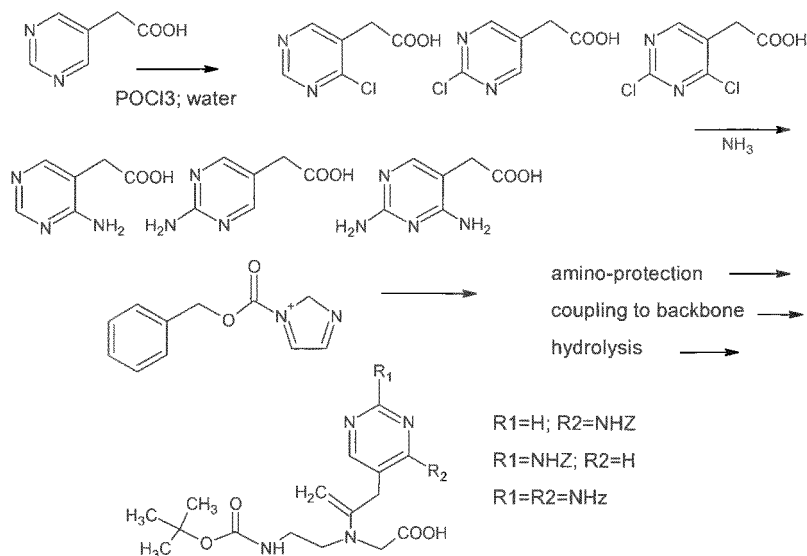
AP-DexVS70-PNA, 5hrs:

PNA/DexVS70: 2.0
 AP/DexVS70: 2.4

[0220] Due to these results, it is recommended to follow a procedure in which the conjugation time (AP + DexVS70-PNA) is 5 hours.

Example 28. Method of synthesizing mono and 2,4-diamino-pyrimidine-5-yl PNA monomers.

[0221] 2,4-diamino-pyrimidine-5-yl may be introduced into DNA-oligomers by methods known in the art (e.g. S.A. Benner et al., Nucleic Acid Research 24(7): 1308-1313 (1996)). A corresponding PNA oligomer is prepared by chlorinating pyrimidine-5-acetic acid to yield 2-chloro-pyrimidine-5-acetic acid, 4-chloro-pyrimidine-5-acetic acid, and 2,4-dichloro-pyrimidine-5-acetic acid. Separation of isomers, followed by high temperature and pressure treatment with ammonia, gives the three corresponding amino-pyrimidine derivatives (see Figure 7). The amino-pyrimidine derivatives are separated and amino-protected, then coupled to a protected PNA backbone ester. Ester hydrolysis results in PNA monomers for production of PNA oligomers containing 2-amino; 4-amino; and/or 2,4-diamino pyrimidine-5-yl bases.



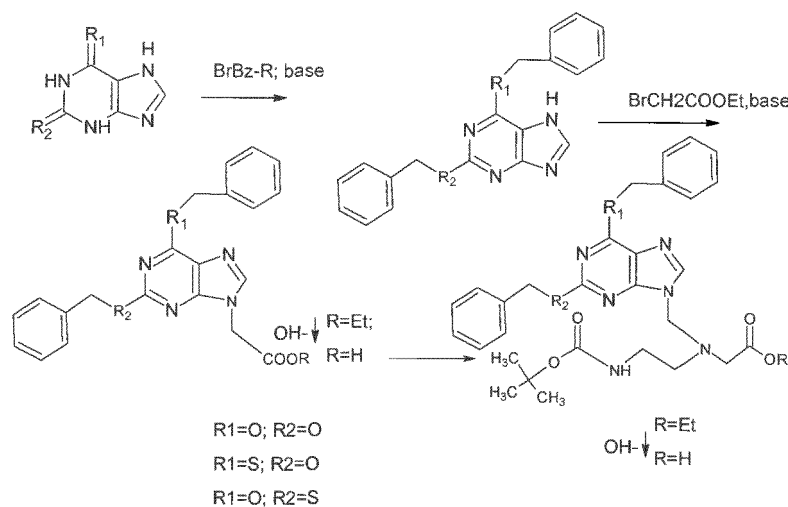
Example 29. Synthesis of xanthine and thio-xanthine-coupled PNA monomers.

[0222] Xanthine, 2-thio-xanthine, and 6-thio-xanthine are commercially available, for instance, from ScienceLab.com.

Further, S.A. Benner et al., Nucleic Acid Research 24(7): 1308-1313 (1996) teaches the preparation of a xanthosine-DNA monomer, including a less acidic and preferable 7-deaza analog, and notes the preferred protection of both oxygens during solid phase synthesis.

[0223] Xanthine PNA-monomers, as well as 2-thio and 6-thio xanthine monomers, are prepared by:

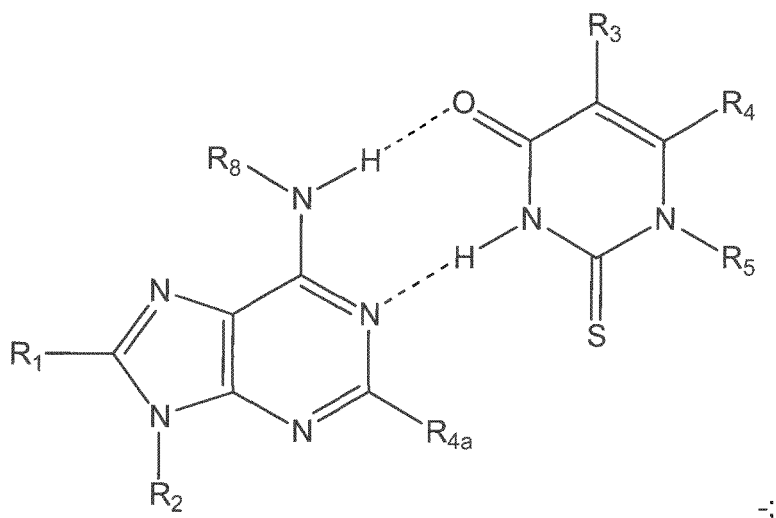
1. Protecting both oxygens or both oxygen and sulphur with appropriate protection groups such as (possibly substituted) benzyl.
2. Alkylating at N-9 with ethyl bromacetate. (Separating N-7 alkylated byproduct.)
3. Hydrolyzing the ethyl ester.
4. HBTU or Carbodiimide-mediated coupling of the nucleobase-acids to 2-Boc-aminoethyl-ethylglycinate.
5. Hydrolyzing the resulting monomer ester to the monomer free acid.
6. The resulting monomers may be used in Merrifield solid phase synthesis of xanthine, 2-thio-xanthine and 6-thio-xanthine-containing PNAs.



Claims

1. A composition comprising a pair of PNA oligomers which

(i) hybridize to each other through base-pairing that comprises more than one of the following combinations :



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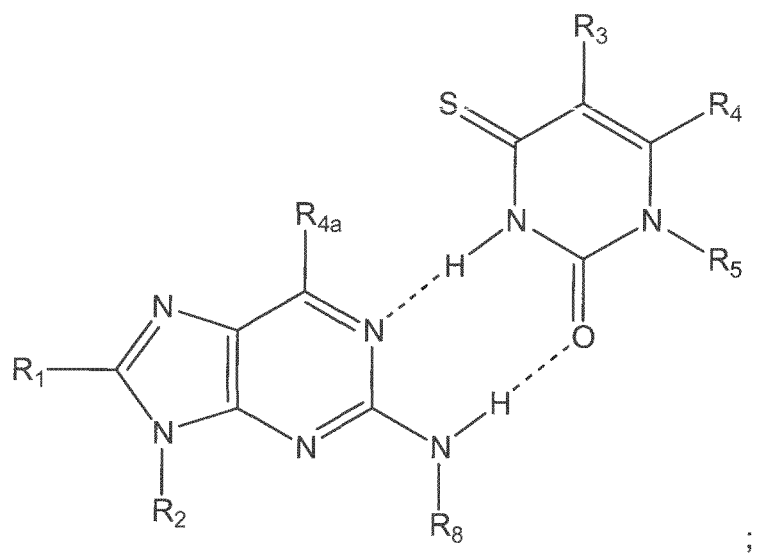
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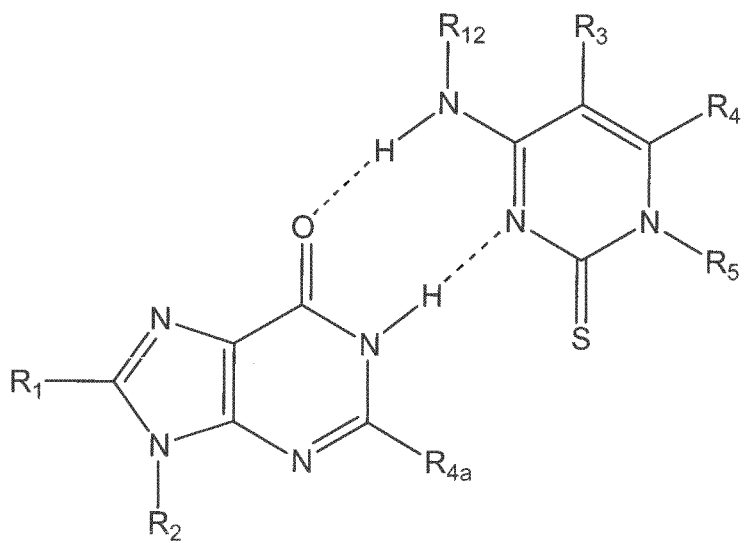
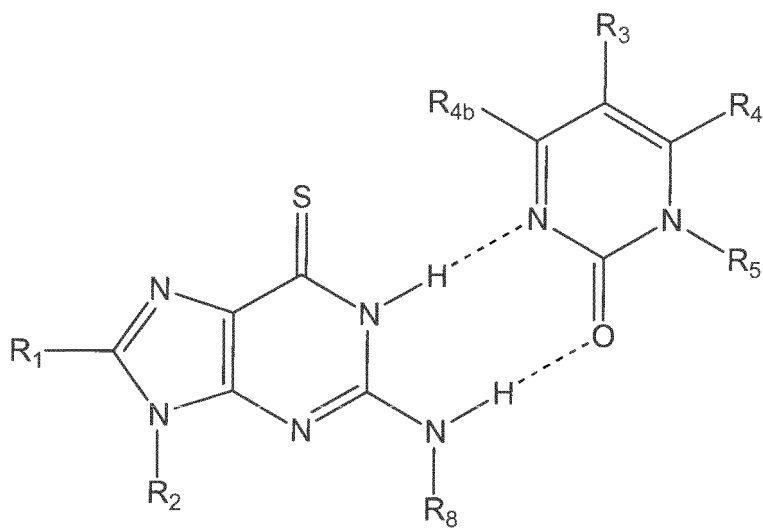
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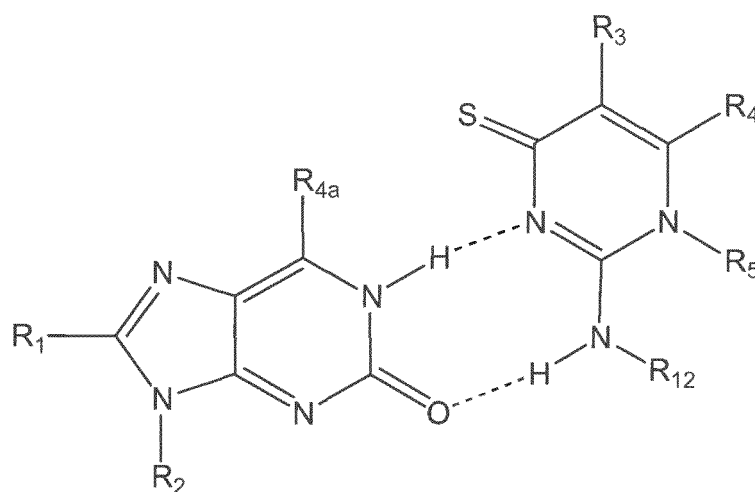
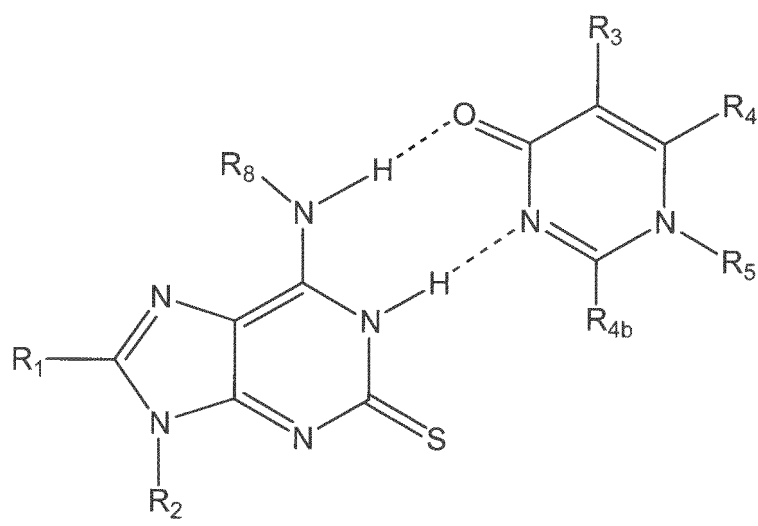
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;



;



wherein:

R₁ is chosen from hydrogen, halogen, optionally substituted (C₁-C₄)-alkyl, -NR₆R₇, optionally substituted (C₁-C₄)-alkoxy, optionally substituted (C₃-C₆)-cycloalkyl, linker, a polyamide backbone, and linker bound to a polyamide backbone;

R₂ is chosen from hydrogen, optionally substituted (C₁-C₄)-alkyl, -NR₆R₇, optionally substituted (C₁-C₄)-alkoxy, optionally substituted (C₃-C₆)-cycloalkyl, linker, a polyamide backbone, and linker bound to a polyamide backbone;

R₃ is group chosen from hydrogen, halogen, optionally substituted (C₁-C₂₀)-alkyl, -NR₆R₇, optionally substituted (C₁-C₂₀)-alkoxy, optionally substituted (C₃-C₈)-cycloalkyl, linker, a polyamide backbone, and linker bound to a polyamide backbone;

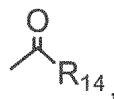
R₄ is chosen from hydrogen, halogen, optionally substituted (C₁-C₄)-alkyl, -NR₆R₇, optionally substituted (C₁-C₄)-alkoxy, optionally substituted (C₃-C₆)-cycloalkyl, linker, a polyamide backbone, and linker bound to a polyamide backbone;

R_{4a} and R_{4b} can be identical or different, and are chosen from hydrogen, fluorine and chlorine.

R₅ is chosen from hydrogen, optionally substituted (C₁-C₄)-alkyl, -NR₆R₇, optionally substituted (C₁-C₄)-alkoxy, optionally substituted (C₃-C₆)-cycloalkyl, linker, a polyamide backbone, and linker bound to a polyamide backbone;

R₆ and R₇ can be identical or different, and are chosen from hydrogen and optionally substituted (C₁-C₂₀)-alkyl;

R₈, R₁₂, can be identical or different, and are chosen from hydrogen, halogen,



optionally substituted (C₁-C₂₀)-alkoxy, and optionally substituted (C₃-C₈)-cycloalkyl, and optionally substituted (C₁-C₂₀)-alkyl, wherein R₁₄ is chosen from hydrogen, optionally substituted (C₁-C₂₀)-alkyl, optionally substituted (C₁-C₂₀)-alkoxy, and optionally substituted (C₃-C₈)-cycloalkyl; and

(ii) do not interfere with RNA or DNA.

2. The composition according to claim 1, wherein R₁, and R₃ each is hydrogen.

3. The composition according to claim 1, wherein R₂ and R₅ can be identical or different and are chosen from linker, a polyamide backbone, and linker bound to a polyamide backbone

4. The composition according to claim 1, wherein:

R₁, R₃, and R₄ each is hydrogen;

R₂ and R₅ can be identical or different and are chosen from a polyamide backbone and linker bound to a polyamide backbone.

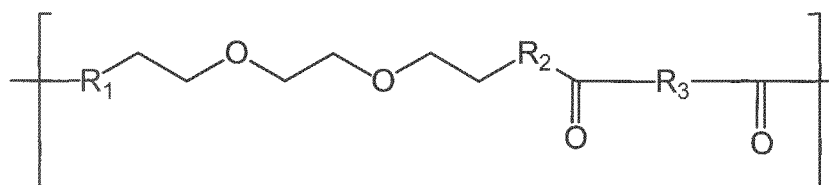
5. The composition of claim 1, comprising a polymer selected from polysaccharides, pullulans, chitins and chitosans, derivatized cellulosics, hydroxylated starch, carrageenans, alginates, and agarose; synthetic polysaccharides such as ficoll and carboxymethylated ficoll; vinyl polymers; poly ethylene glycol (PEG) or polypropylene glycol or poly(ethylene oxide-co-propylene oxides) containing polymer backbones including linear, comb-shaped or hyperbranched polymers and dendrimers, including branched PAMAM-dendrimers; poly amino acids including polylysines, polyglutamic acid, polyurethanes, poly(ethylene imines), pluriol; proteins including albumins, immunoglobulins, and virus-like proteins (VLP), and polynucleotides such as DNA, RNA, LNA.

6. The composition of claim 5 wherein the polymer is a dextran.

7. The composition of claim 1, comprising a linker.

8. The composition according to claim 7, wherein the linker is chosen from a molecule comprising polyethylene glycol and a molecule comprising at least two units according to the Formula I:

Formula I

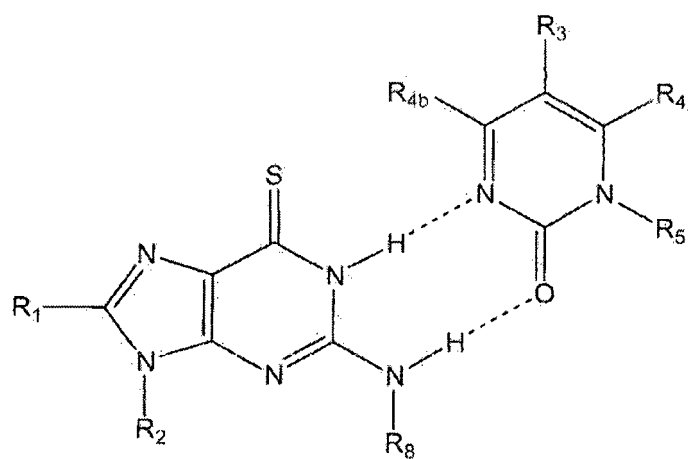
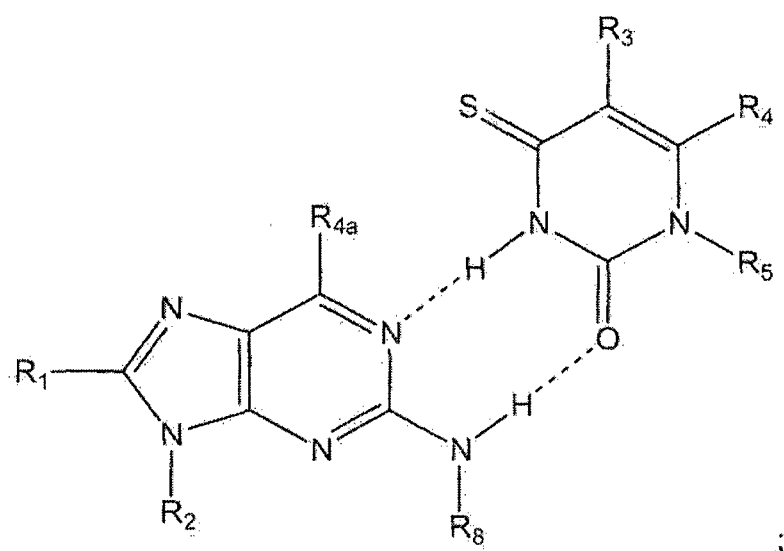
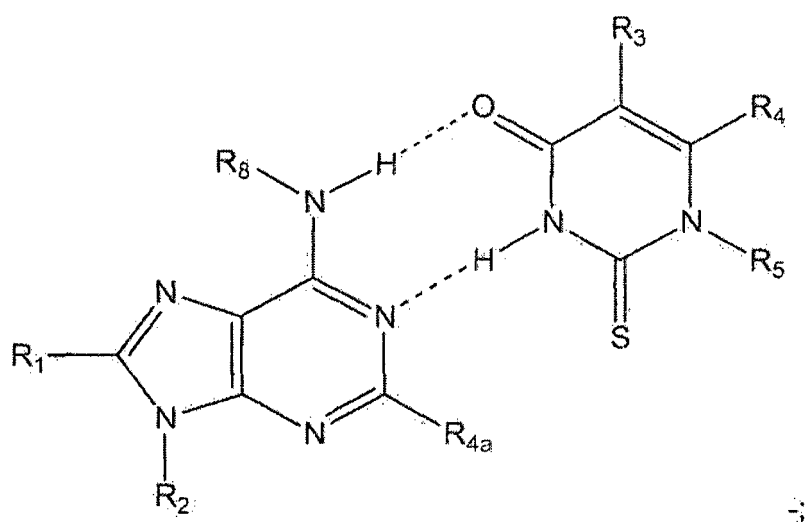


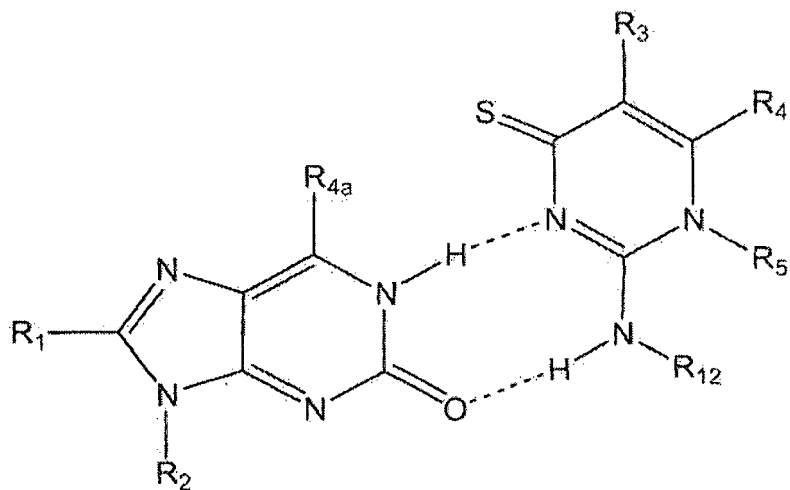
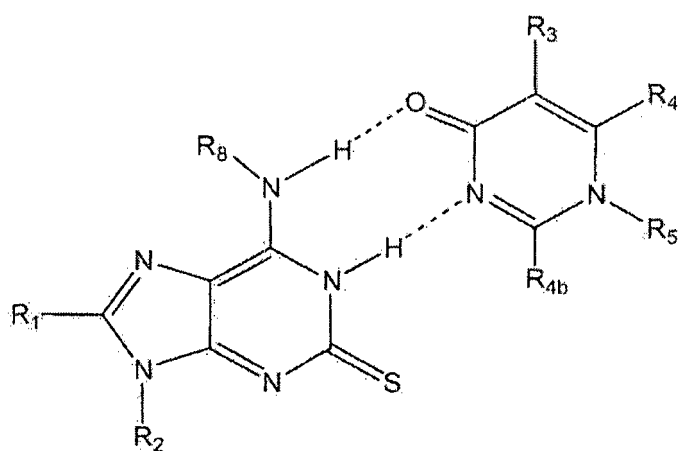
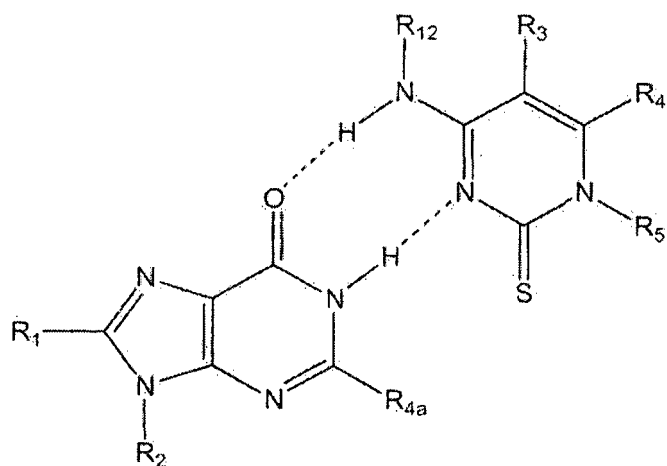
wherein R₁ and R₂ comprise NH or O, and R₃ comprises methyl, ethyl, propyl, CH₂-O-CH₂, and (CH₂-O-CH₂)₂.

Patentansprüche

1. Zusammensetzung umfassend ein Paar von PNA-Oligomeren, welche

(i) durch Basenpaarung aneinander hybridisieren, welche mehr als eine der folgenden Kombinationen umfasst:





wobei:

R_1 ausgewählt ist aus Wasserstoff, Halogen, gegebenenfalls substituiertem (C₁-C₄)-Alkyl, -NR₆R₇, gegebenenfalls substituiertem (C₁-C₄)-Alkoxy, gegebenenfalls substituiertem (C₃-C₆)-Cycloalkyl, Linker, einem Polyamidgrundgerüst, und Linker gebunden an ein Polyamidgrundgerüst;
 R_2 ausgewählt ist aus Wasserstoff, gegebenenfalls substituiertem (C₁-C₄)-Alkyl, -NR₆R₇, gegebenenfalls substituiertem (C₁-C₄)-Alkoxy, gegebenenfalls substituiertem (C₃-C₆)-Cycloalkyl, Linker, einem Polyamidgrundgerüst, und Linker gebunden an ein Polyamidgrundgerüst;

R_3 eine Gruppe ist, ausgewählt aus Wasserstoff, Halogen, gegebenenfalls substituiertem (C_1 - C_{20})-Alkyl, $-NR_6R_7$, gegebenenfalls substituiertem (C_1 - C_{20})-Alkoxy, gegebenenfalls substituiertem (C_3 - C_8)-Cycloalkyl, Linker, einem Polyamidgrundgerüst, und Linker gebunden an ein Polyamidgrundgerüst;

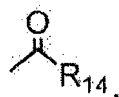
R_4 ausgewählt ist aus Wasserstoff, Halogen, gegebenenfalls substituiertem (C_1 - C_4)-Alkyl, $-NR_6R_7$, gegebenenfalls substituiertem (C_1 - C_4)-Alkoxy, gegebenenfalls substituiertem (C_3 - C_6)-Cycloalkyl, Linker, einem Polyamidgrundgerüst, und Linker gebunden an ein Polyamidgrundgerüst;

R_{4a} und R_{4b} identisch oder verschieden sein können und ausgewählt sind aus Wasserstoff, Fluor und Chlor;

R_5 ausgewählt ist aus Wasserstoff, gegebenenfalls substituiertem (C_1 - C_4)-Alkyl, $-NR_6R_7$, gegebenenfalls substituiertem (C_1 - C_4)-Alkoxy, gegebenenfalls substituiertem (C_3 - C_6)-Cycloalkyl, Linker, einem Polyamidgrundgerüst, und Linker gebunden an ein Polyamidgrundgerüst;

R_6 und R_7 identisch oder verschieden sein können und ausgewählt sind aus Wasserstoff und gegebenenfalls substituiertem (C_1 - C_{20})-Alkyl;

R_8 , R_{12} identisch oder verschieden sein können und ausgewählt sind aus Wasserstoff, Halogen,



gegebenenfalls substituiertem (C_1 - C_{20})-Alkoxy und gegebenenfalls substituiertem (C_3 - C_8)-Cycloalkyl, und gegebenenfalls substituiertem (C_1 - C_{20})-Alkyl, wobei R_{14} ausgewählt ist aus Wasserstoff, gegebenenfalls substituiertem (C_1 - C_{20})-Alkyl, gegebenenfalls substituiertem (C_1 - C_{20})-Alkoxy und gegebenenfalls substituiertem (C_3 - C_8)-Cycloalkyl; und

(ii) nicht mit RNA oder DNA interferieren.

2. Zusammensetzung gemäß Anspruch 1, wobei R_1 und R_3 jeweils Wasserstoff ist.

3. Zusammensetzung gemäß Anspruch 1, wobei R_2 und R_5 identisch oder verschieden sein können und ausgewählt sind aus Linker, einem Polyamidgrundgerüst, und Linker gebunden an ein Polyamidgrundgerüst.

4. Zusammensetzung gemäß Anspruch 1, wobei:

R_1 , R_3 und R_4 jeweils Wasserstoff ist;

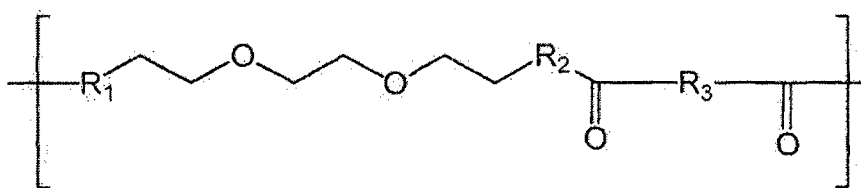
R_2 und R_5 identisch oder verschieden sein können und ausgewählt sind aus einem Polyamidgrundgerüst und Linker gebunden an ein Polyamidgrundgerüst.

5. Zusammensetzung nach Anspruch 1, umfassend ein Polymer ausgewählt aus Polysacchariden, Pullulanen, Chitinen und Chitosanen, derivatisierten Celluloseerzeugnissen, hydroxylierter Stärke, Carregenenen, Alginaten und Agarose; synthetischen Polysacchariden wie Ficoll und carboxymethyliertes Ficoll; Vinylpolymeren; Polyethylenglycol (PEG) oder Polypropylenglycol oder Poly(ethylenoxid-co-propylenoxide) enthaltenden Polymergrundgerüsten einschließlich linearer, kammförmiger oder hypervverzweigter Polymere und Dendrimere, einschließlich verzweigter PAMAM-Dendrimere; Polyaminosäuren einschließlich Polylysine, Polyglutaminsäure, Polyurethanen, Poly(ethylenimin), Pluriol; Proteinen einschließlich Albumine, Immunglobulinen und virusartigen Proteinen (virus-like proteins, VLP) und Polynucleotiden wie DNA, RNA und LNA.

6. Zusammensetzung nach Anspruch 5, wobei das Polymer ein Dextran ist.

7. Zusammensetzung nach Anspruch 1, umfassend einen Linker.

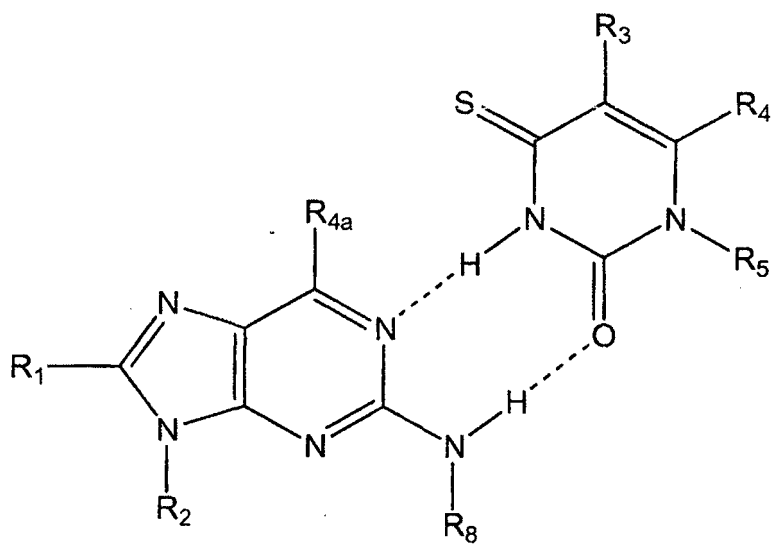
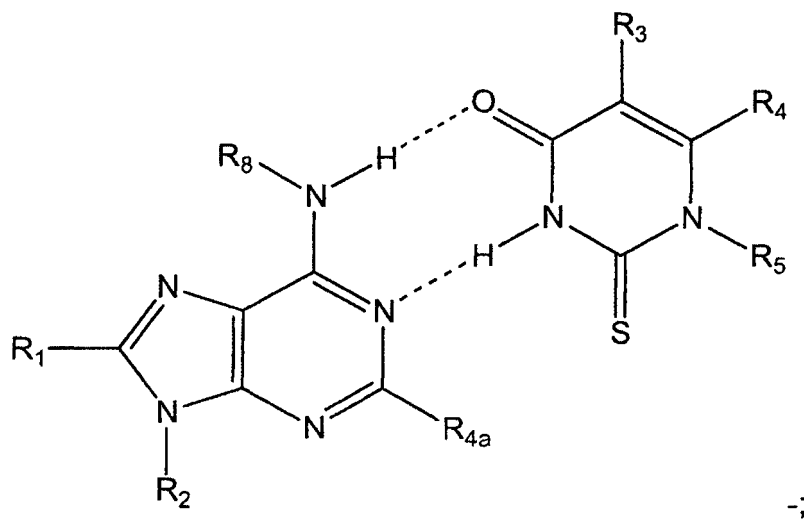
8. Zusammensetzung gemäß Anspruch 7, wobei der Linker ausgewählt ist aus einem Molekül, das Polyethylenglycol umfasst, und einem Molekül, das wenigstens zwei Einheiten gemäß der Formel I umfasst:

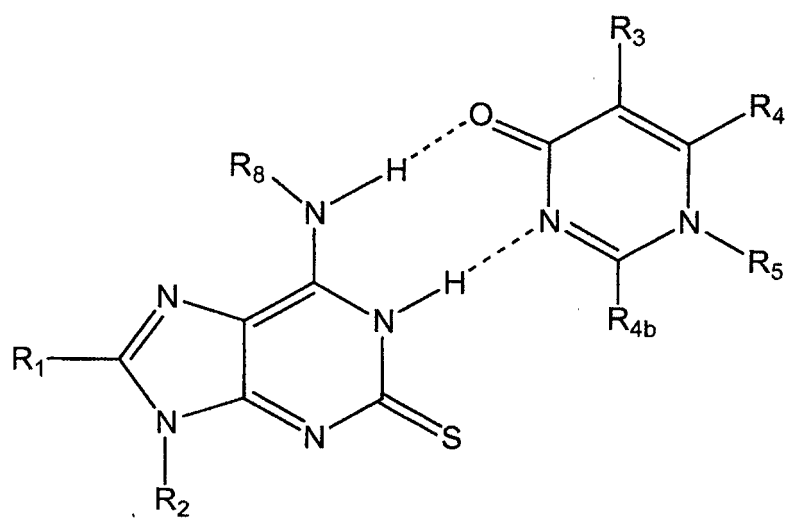
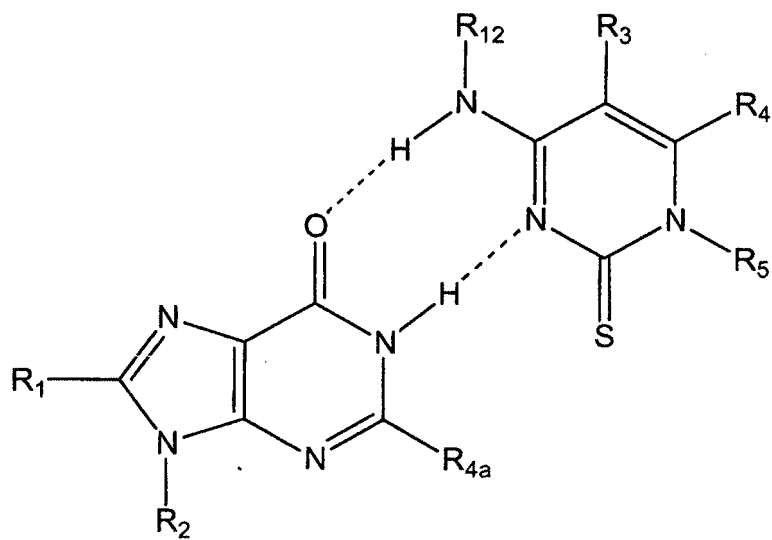
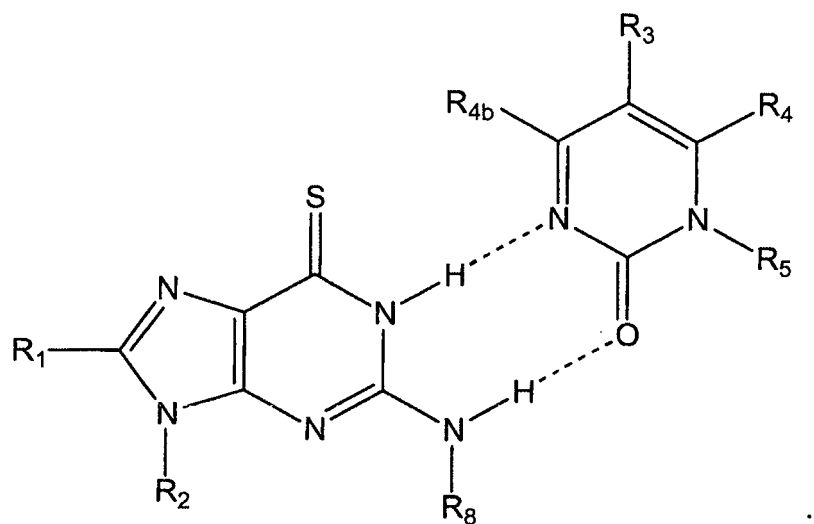
Formel I

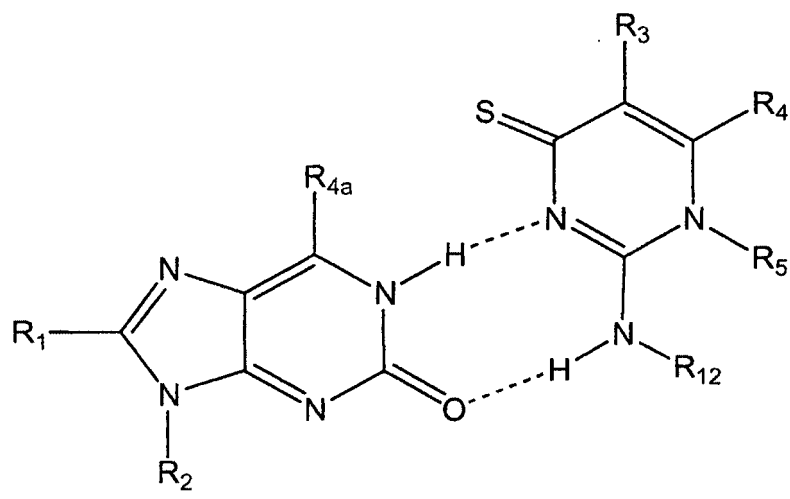
wobei R_1 und R_2 NH oder O umfassen und R_3 Methyl, Ethyl, Propyl, $\text{CH}_2\text{-O-CH}_2$ und $(\text{CH}_2\text{-O-CH}_2)_2$ umfasst.

Revendications**1.** Composition comprenant une paire d'oligomères PNA qui

(i) s'hybrident entre eux par un appariement de bases comprenant plus d'une des combinaisons suivantes :







dans lesquelles :

R₁ est choisi parmi un hydrogène, un halogène, un (C₁-C₄)-alkyle éventuellement substitué, -NR₆R₇, un (C₁-C₄)-alcoyle éventuellement substitué, un (C₃-C₆)-cycloalkyle éventuellement substitué, un liant, un squelette polyamide, et un liant lié à un squelette polyamide ;

R₂ est choisi parmi un hydrogène, un (C₁-C₄)-alkyle éventuellement substitué, -NR₆R₇, un (C₁-C₄)-alcoyle éventuellement substitué, un (C₃-C₆)-cycloalkyle éventuellement substitué, un liant, un squelette polyamide, et un liant lié à un squelette polyamide ;

R₃ est un groupe choisi parmi un hydrogène, un halogène, un (C₁-C₂₀)-alkyle éventuellement substitué, -NR₆R₇, un (C₁-C₂₀)-alcoyle éventuellement substitué, un (C₃-C₈)-cycloalkyle éventuellement substitué, un liant, un squelette polyamide, et un liant lié à un squelette polyamide ;

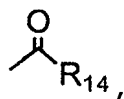
R₄ est choisi parmi un hydrogène, un halogène, un (C₁-C₄)-alkyle éventuellement substitué, -NR₆R₇, un (C₁-C₄)-alcoyle éventuellement substitué, un (C₃-C₆)-cycloalkyle éventuellement substitué, un liant, un squelette polyamide, et un liant lié à un squelette polyamide ;

R_{4a} et R_{4b} peuvent être identiques ou différents et sont choisis parmi un hydrogène, un fluor, et un chlore ;

R₅ est choisi parmi un hydrogène, un (C₁-C₄)-alkyle éventuellement substitué, -NR₆R₇, un (C₁-C₄)-alcoyle éventuellement substitué, un (C₃-C₆)-cycloalkyle éventuellement substitué, un liant, un squelette polyamide, et un liant lié à un squelette polyamide ;

R₆ et R₇ peuvent être identiques ou différents et sont choisis parmi un hydrogène et un (C₁-C₂₀)-alkyle éventuellement substitué ;

R₈, R₁₂ peuvent être identiques ou différents et sont choisis parmi un hydrogène, un halogène,



un (C₁-C₂₀)-alcoyle éventuellement substitué, un (C₃-C₈)-cycloalkyle éventuellement substitué, et un (C₁-C₂₀)-alkyle éventuellement substitué, dans lequel R₁₄ est choisi parmi un hydrogène, un (C₁-C₂₀)-alkyle éventuellement substitué, un (C₁-C₂₀)-alcoyle éventuellement substitué, et un (C₃-C₈)-cycloalkyle éventuellement substitué, et

(ii) n'interfèrent pas avec l'ARN ou l'ADN.

2. Composition selon la revendication 1, dans laquelle R₁ et R₃ sont chacun un hydrogène.

3. Composition selon la revendication 1, dans laquelle R₂ et R₅ peuvent être identiques ou différents et sont choisis parmi un liant, un squelette polyamide, et un liant lié à un squelette polyamide.

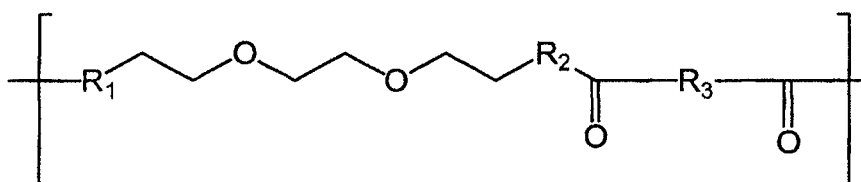
4. Composition selon la revendication 1, dans laquelle :

R_1 , R_3 et R_4 sont chacun un hydrogène ;

R_2 et R_5 peuvent être identiques ou différents et sont choisis parmi un squelette polyamide, et un liant lié à un squelette polyamide.

- 5 5. Composition selon la revendication 1, comprenant un polymère sélectionné parmi les polysaccharides, les pullulanes, les chitines et les chitosanes, les dérivés cellulosiques, l'amidon hydroxylé, les carraghénanes, les alginates, et l'agarose ; les polysaccharides synthétiques tels que le ficoll et le ficoll carboxyméthylé ; les polymères de vinyle ; les squelettes polymères contenant du polyéthylène glycol (PEG) ou du polypropylène glycol ou des polyoxydes (éthylène-co-propylène) y compris les polymères et dendrimères linéaires, en forme de peigne ou hyperramifiés, y compris les dendrimères PAMAM ramifiés ; les poly(acides aminés) y compris les polylysines, le poly(acide glutamique), les polyuréthanes, les polyaziridines, le pluriol ; les protéines y compris les albumines, les immunoglobulines et les protéines pseudo-virales (VLP), et les polynucléotides tels que l'ADN, l'ARN et le LNA.
- 10 6. Composition selon la revendication 5, dans laquelle le polymère est un dextrane.
- 15 7. Composition selon la revendication 1, comprenant un liant.
- 20 8. Composition selon la revendication 7, dans laquelle le liant est choisi parmi une molécule comprenant du polyéthylène glycol et une molécule comprenant au moins deux unités selon la Formule I :

Formule I



25 dans laquelle R_1 et R_2 comprennent NH ou O, et R_3 comprend les groupes méthyle, éthyle, propyle, $\text{CH}_2\text{-O-CH}_2$, et $(\text{CH}_2\text{-O-CH}_2)_2$.

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Figure 1

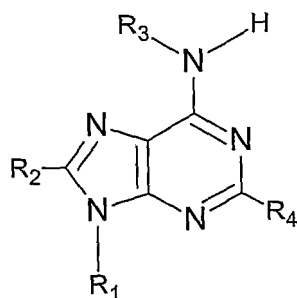


Figure 1a, Adenine, A

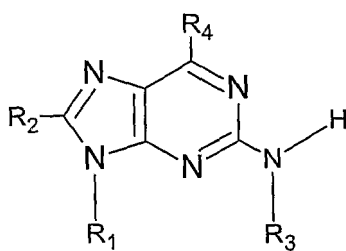


Figure 1b, isoAdenine, isoA

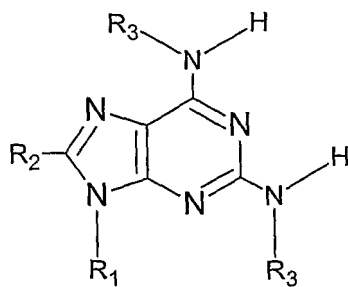


Figure 1c, Diaminopurine, D

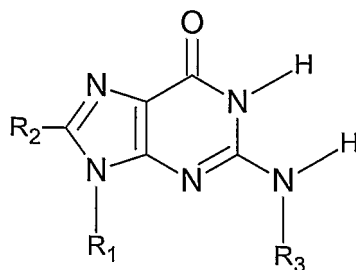


Figure 1d, Guanine, G

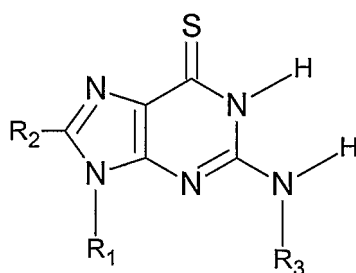


Figure 1e, ThioGuanine, Gs

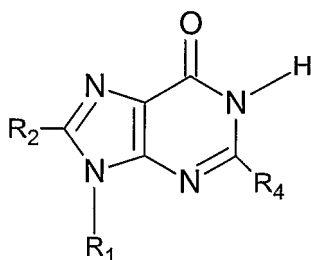


Figure 1f, Inosine, I

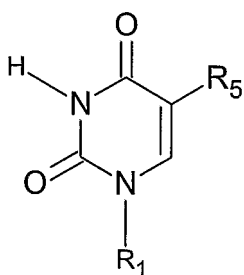


Figure 1g, Uracil, U

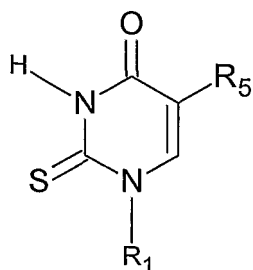


Figure 1h, 2-ThioUracil, U2s

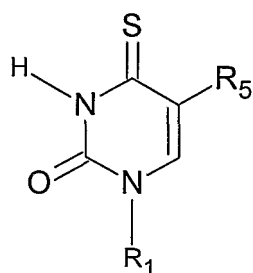


Figure 1i, 4-ThioUracil, U4s

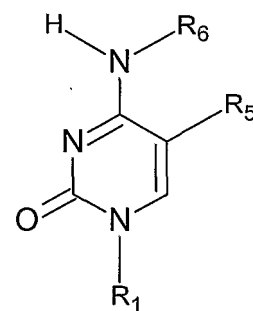


Figure 1j, Cytosine, C

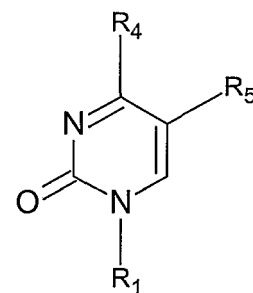


Figure 1k, 2-oxo-Pyrimidine, Py-2o

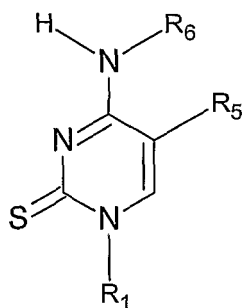


Figure 1l, ThioCytosine, Cs

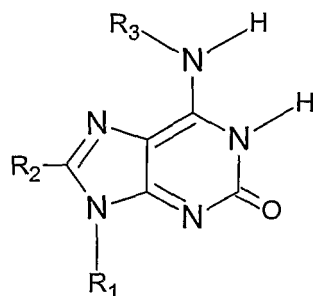


Figure 1m, isoGuanine, isoG

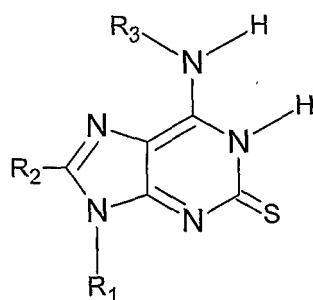


Figure 1n, isothioGuanine, isoGs

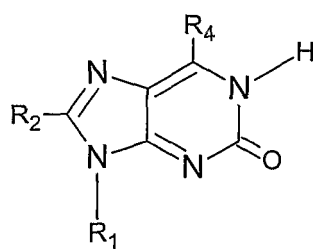


Figure 1o, 2-oxoPurine, Pu-2o

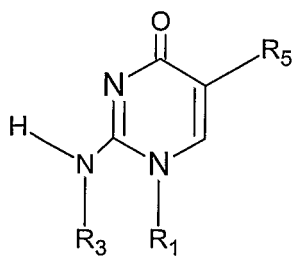


Figure 1p, isoCytosine, isoC

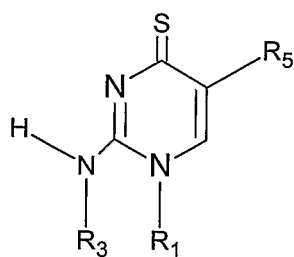


Figure 1q, isothioCytosine, isoCs

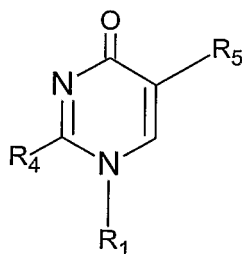


Figure 1r, 4-oxoPyrimidine, Py-4o

Figure 2

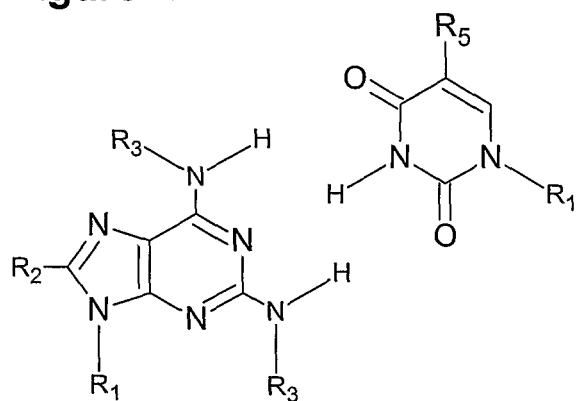


Figure 2a Diaminopurine-Uracil

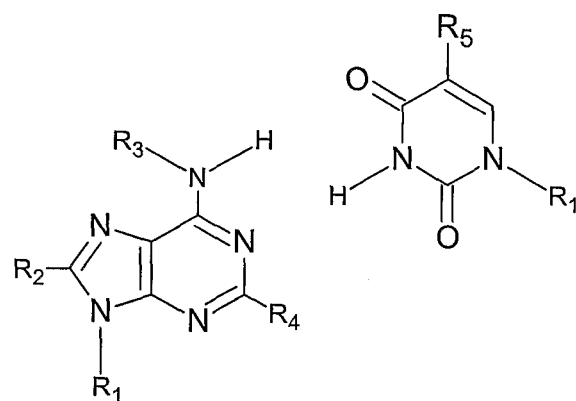


Figure 2b Adenine-Uracil

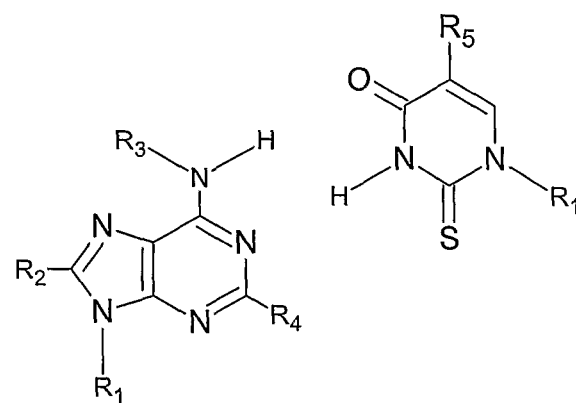


Figure 2c Adenine-2-thioUracil

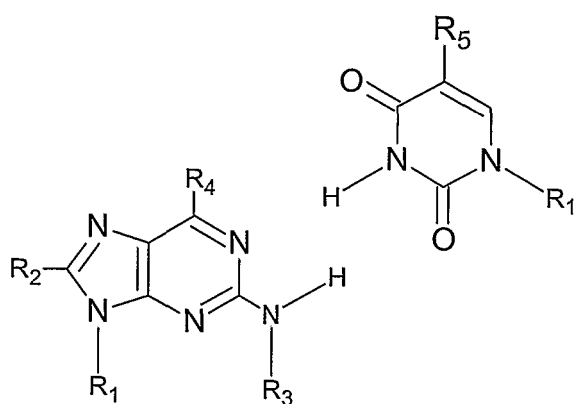


Figure 2d isoAdenine-Uracil

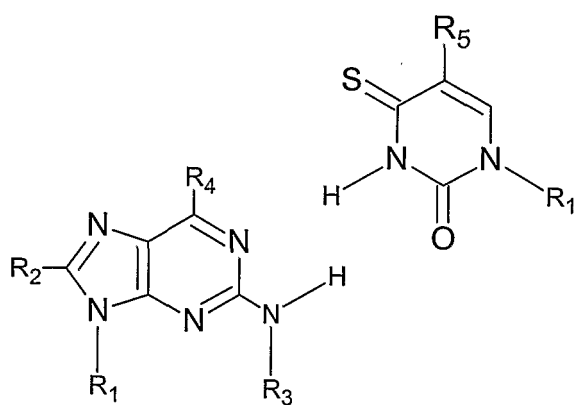


Figure 2e isoAdenine-4thioUracil

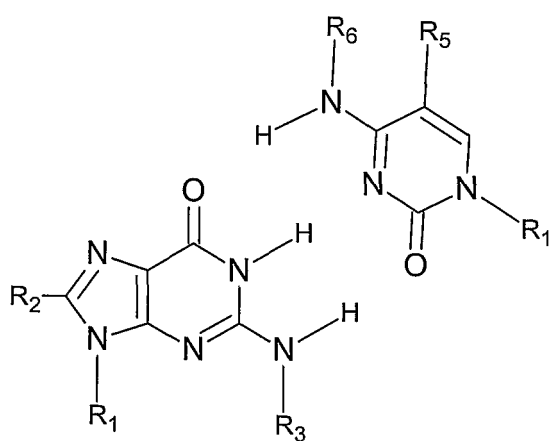


Figure 2f Guanine-Cytosine

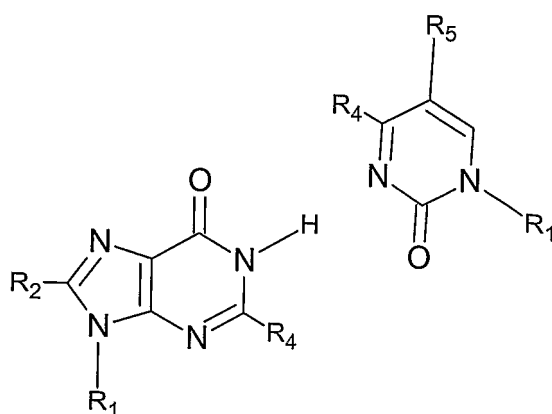


Figure 2g Guanine-2-oxo-Pyrimidine

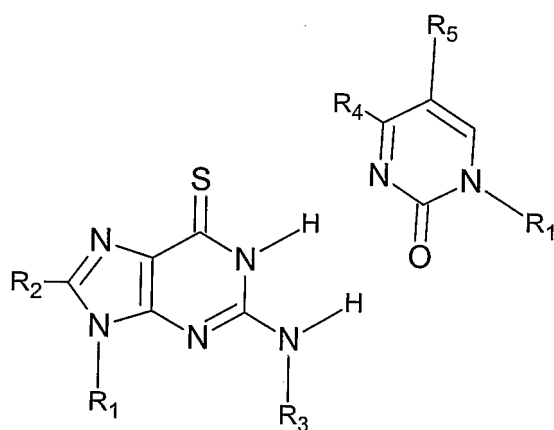


Figure 2h ThioGuanine-2-oxo-Pyrimidine

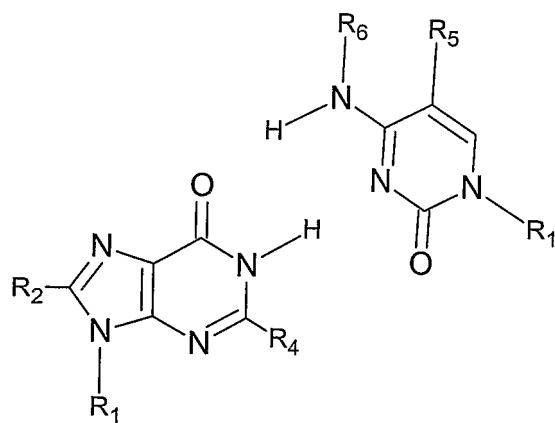


Figure 2i Inosine-Cytosine

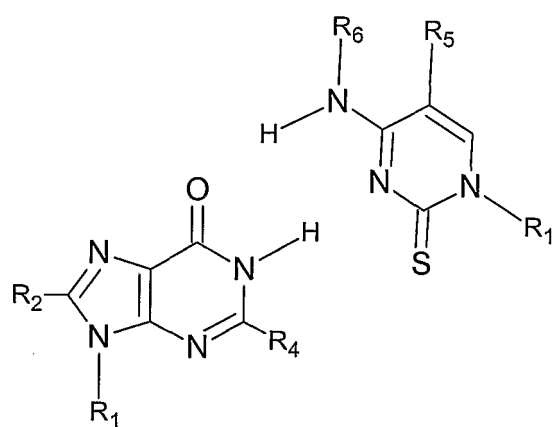


Figure 2j Inosine-ThioCytosine

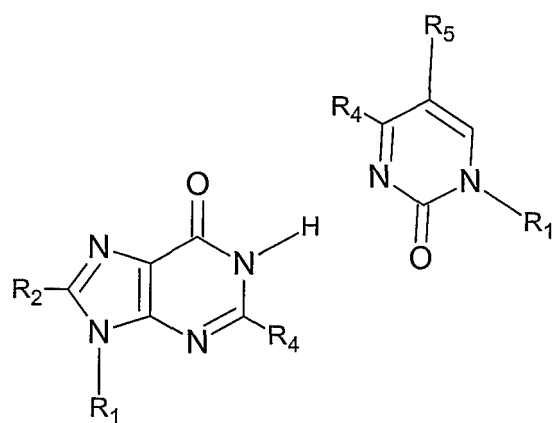


Figure 2k Inosine-2-oxo-Pyrimidine

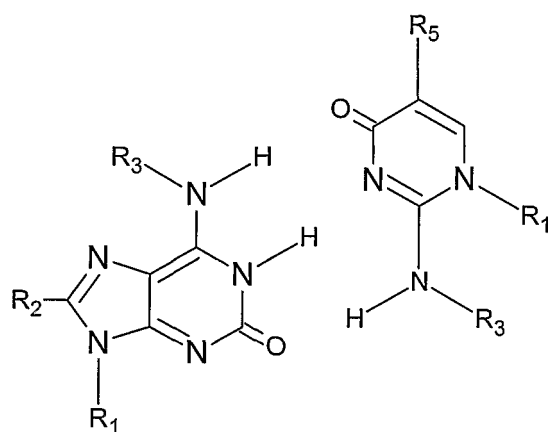


Figure 2l IsoGuanine-isoCytosine

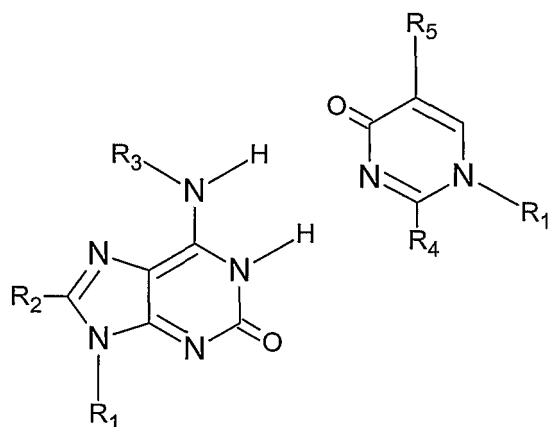


Figure 2m isoGuanine-4-oxo-Pyrimidine

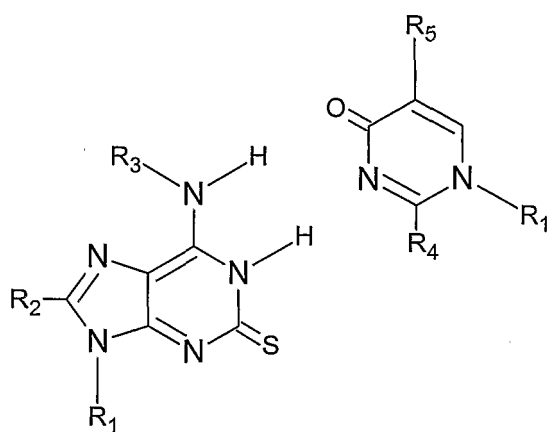


Figure 2n isothioGuanine-4-oxo-pyrimidine

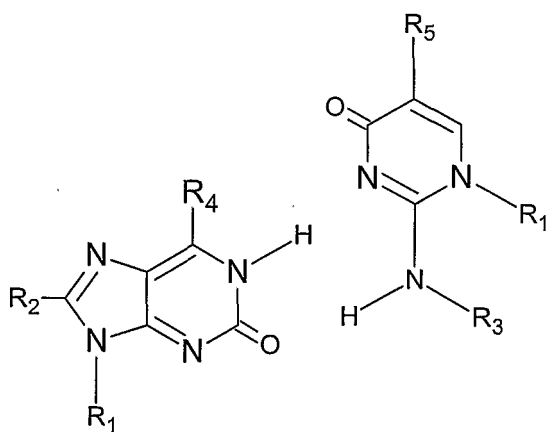


Figure 2o 2-oxo-Purine-isoCytosine

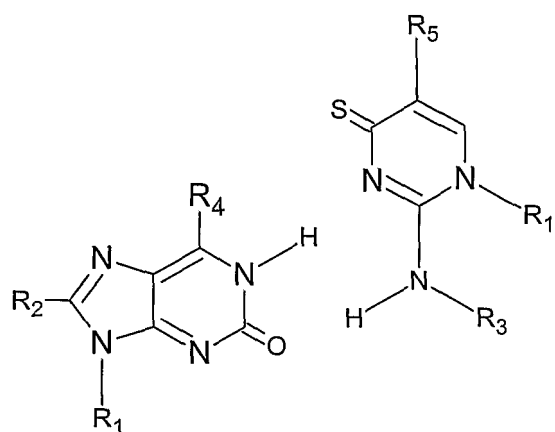


Figure 2p 2-oxo-Purine-isothioCytosine

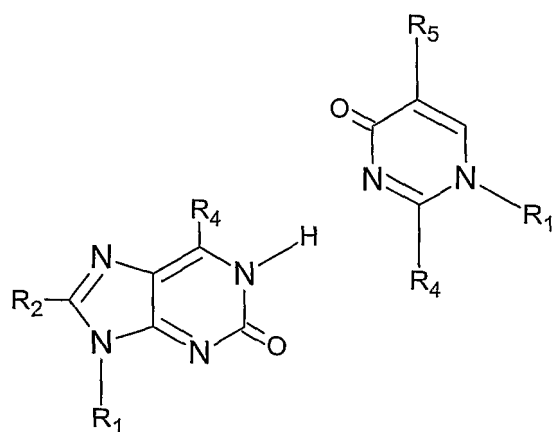


Figure 2q 2-oxo-Purine-4-oxo-Pyrimidine

Figure 3

Base Pairs	Diaminopurine	Adenine
Uracil	3 H-bonds	2 H-bonds
2-ThioUracil	repulsion	2 H-bonds

Base Pairs	Diaminopurine	isoAdenine
Uracil	3 H-bonds	2 H-bonds
4-ThioUracil	repulsion	2 H-bonds

Base Pairs	Guanine	Inosine
Cytosine	3 H-bonds	2 H-bonds
2-ThioCytosine	repulsion	2 H-bonds

Base Pairs	Cytosine	2-oxo-Pyrimidine
Guanine	3 H-bonds	2 H-bonds
ThioGuanine	repulsion	2 H-bonds

Base Pairs	isoGuanine	2-oxo-Purine
isoCytosine	3 H-bonds	2 H-bonds
isoThioCytosine	repulsion	2 H-bonds

Base Pairs	isoCytosine	4-Oxo-Pyrimidine
isoGuanine	3 H-bonds	2 H-bonds
isoThioGuanine	repulsion	2 H-bonds

Figure 4

Degeneracy1

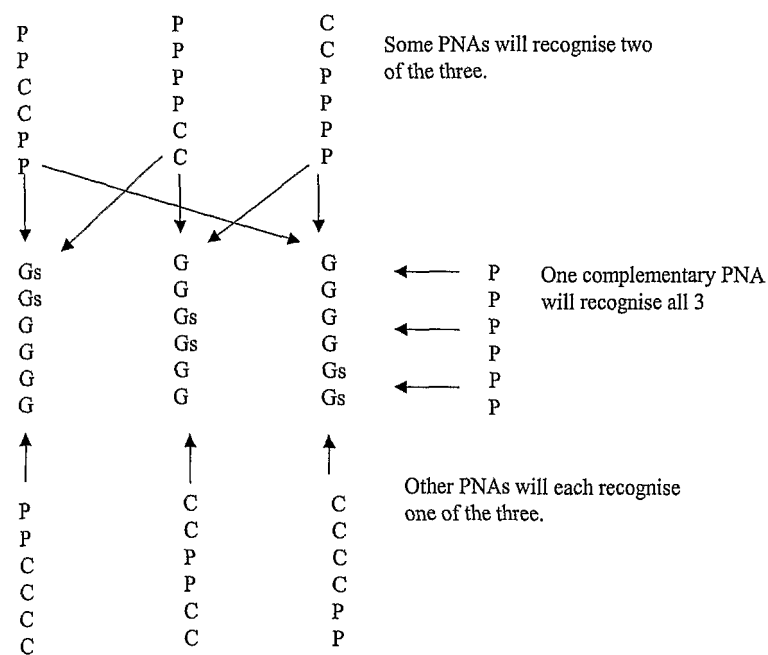


Figure 5

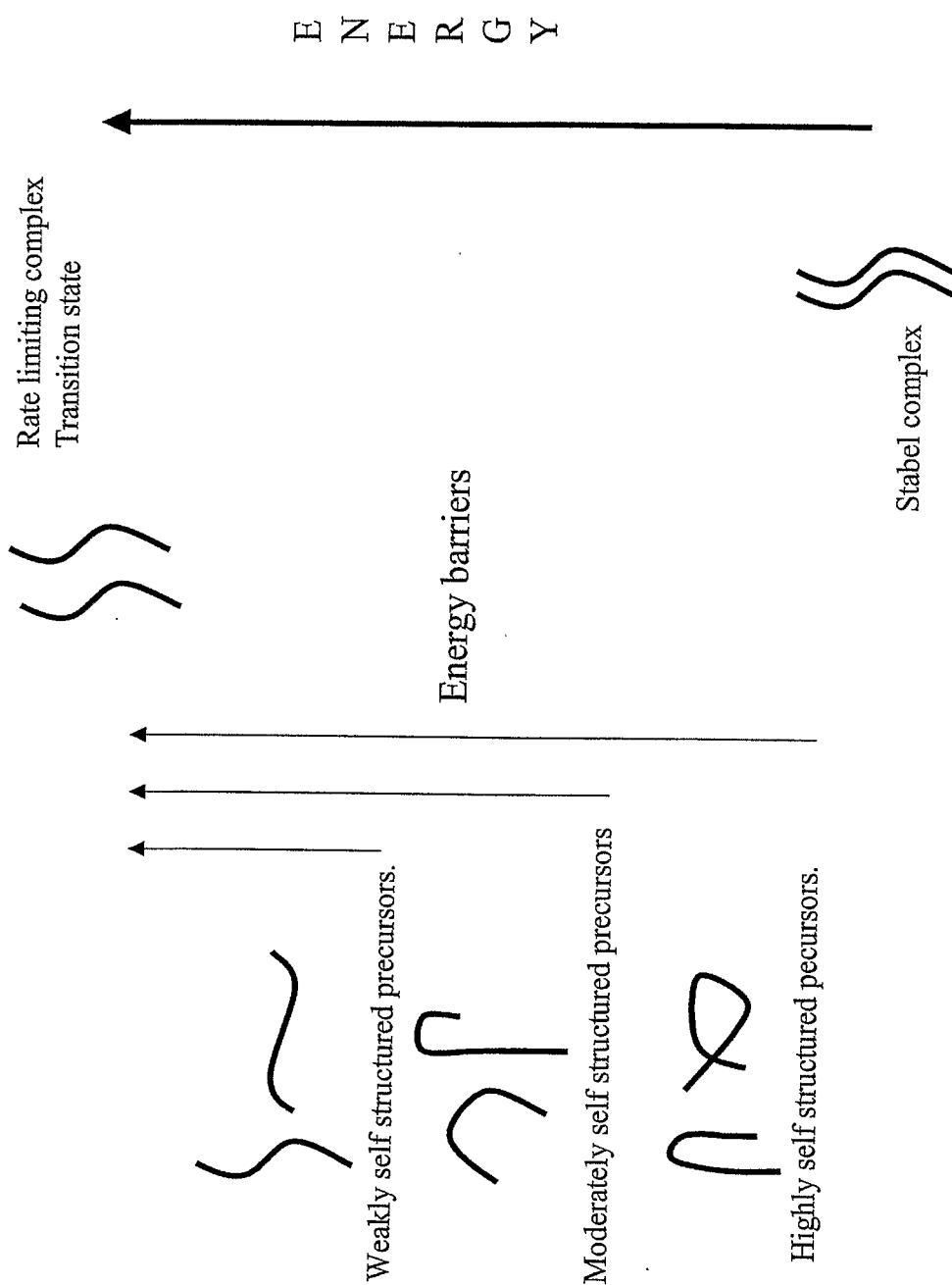
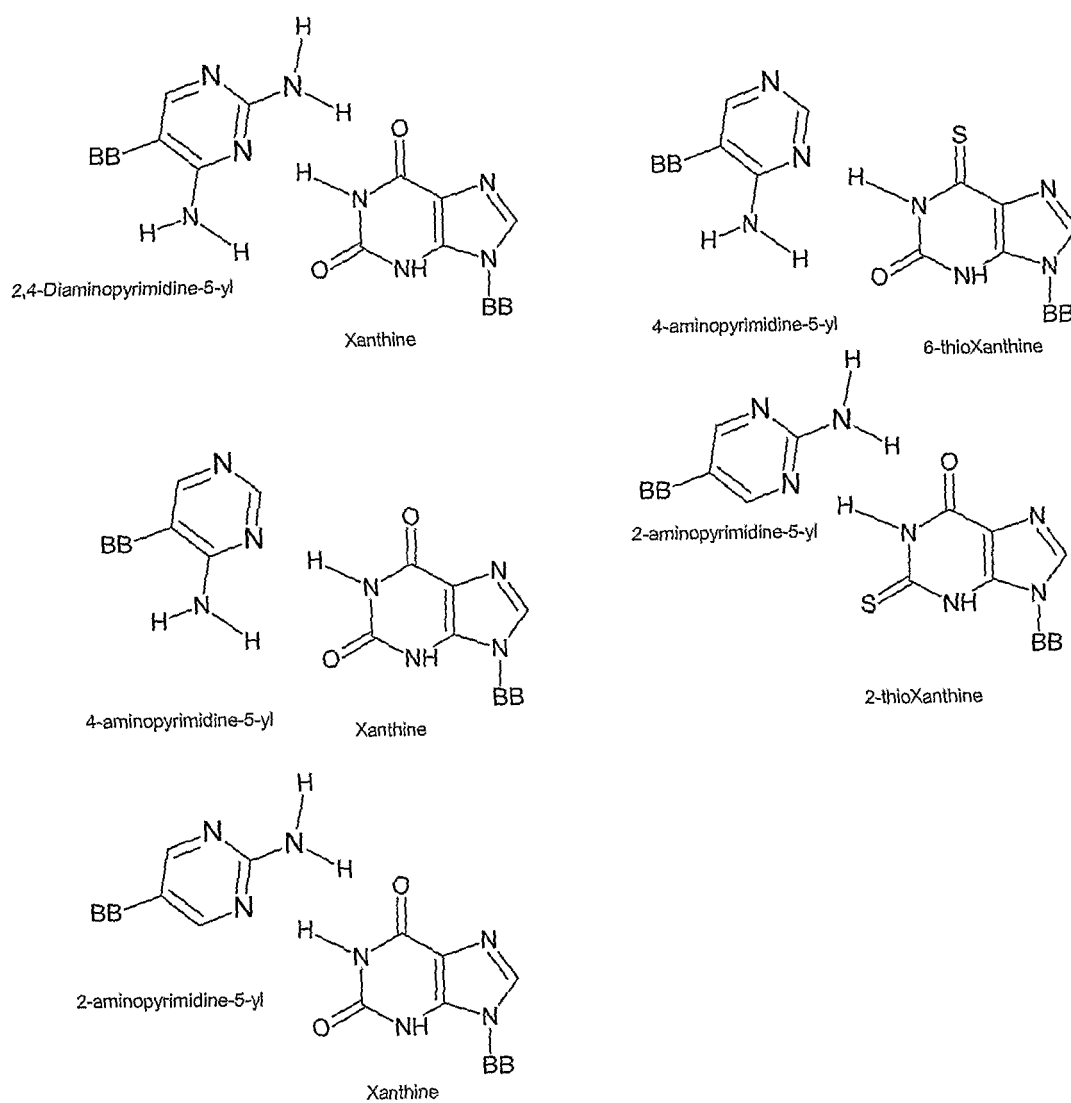


Figure 6

	Adenines	6-aminopurines	2,6-diaminopurines	Guanines	Inosines	6-Thioguanines	isoguanines	iso-2-thioguanines	2-oxo-purines	Uracils	2-thioxouracils	4-thioxouracils	cytosine	2-thioxocytosine	2-oxo-pyrimidines	isocytosine	iso-4-thioxocytosine	4-oxo-pyrimidines
Adenines																		
6-aminopurines																		
2,6-diaminopurines																		
Guanines																		
Inosines																		
6-Thioguanines																		
isoguanines																		
iso-2-thioguanines																		
2-oxo-purines																		
Uracils	2	2	3															
2-thioxouracils	2																	
4-thioxouracils	2																	
cytosine													3	2				
2-thioxocytosine														2				
2-oxo-pyrimidines														2	1	2		
isocytosine																3	2	
iso-4-thioxocytosine																	2	
4-oxo-pyrimidines																	2	1

Figure 7



REFERENCES CITED IN THE DESCRIPTION

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